VOLUME 2 OCTOBER, 1956 No. 4

THE JOURNAL OF GENERAL AND APPLIED MICROBIOLOGY

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Published Quarterly By

THE INSTITUTE OF APPLIED MICROBIOLOGY

THE UNIVERSITY OF TOKYO

TOKYO JAPAN

COMMONWEALTH MYCOLOGICAL INSTITUTE
11 MAR 1957

The Journal of General and Applied Microbiology is issued quarterly by the Institute of Applied Microbiology, University of Tokyo.

The journal is distributed without charge to, preferably in exchange with similar publications from, outstanding scientists and scientific organizations interested in microbiology and related fields.

Correspondence concerning the journal should be addressed to the Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

J. Gen. Appl. Microbiol.
Vol. 2, No. 4, 1956

HEREDITARY VARIATION AND RECOMBINATION IN KOJI-MOLDS (*ASPERGILLUS ORYZAE* AND *ASP. SOJAE*) V. HETEROCARYOSIS

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Received for publication Feb. 1, 1956

The mechanism of variation in certain filamentous fungi with no perfect sexual cycle, such as the Aspergilli and the Penicillia has been mainly attributed to heterocaryosis and mutation. The term "heterocaryosis" refers to the condition where two or more genetically different kinds of nuclei exist in the same cell. This situation is attained by the interweaving of different hyphae, resulting in anastomoses or fusions and the migration of one or more nuclei from one cell to another. The exchanged nuclei divide mitotically, independently, and without nuclear fusion, and thus multinucleate hyphal cells or conidia contain genetically unlike nuclei. This phenomenon was first described in 1884 by the great botanist, DeBary[1]. The detailed demonstration of the occurrence of heterocaryosis in fungi and its significance from the standpoint of the variability of imperfect fungi was first clearly advanced by Hansen and Smith[2] in *Botrytis cinerea* and later developed for many different genera of fungi (Hansen and Smith[3]; Hansen[4]).

Hansen's main concept of heterocaryosis has been confirmed and broadened by Dodge[5], Beadle and Coonradt[6], Baker[7-8], Pontecorvo and Gemmell[9], Pontecorvo[10], Wilhelm[11], Raper and Fennell[12], Buxton[13], etc. The frequently occurring multinucleate condition in hyphal cells and spores of such fungi has suggested the possibility of an irregular distribution of genetically distinct nuclei during cell division or conidial formation.

In our study the following problems on heterocaryosis have been extensively investigated since 1952, using the fungi, *Aspergillus oryzae* and *Asp. sojae*: 1) The effects of cultural conditions[14] and genetic condition[15] on anastomosis, which is a primary requirement for heterocaryosis. 2) The study of genetic markers[16-20] for the production of heterocaryons; what kinds of genetic markers should be used for isolation of heterocaryons? 3) Preservation or stabilization of heterocaryons including the breakdown of synthesized strains under some environmental conditions[17], and the production of stable new heterozygous diploid strains[21]. 4) The phenotype of heterocaryons—morphological and physiological characteristics, dominance relationships between mutant genes and their normal alleles, and heterocaryotic

vigor, in relation to the production of more potent enzyme-producing powers than those of the component strains[22]. 5) Determination of the allelism[17-18] of mimic mutants (nutritionally similar mutants), by using the prototrophic heterocaryon method. 6) Induction of heterocaryosis[23] by means other than anastomosis.

EXPERIMENTAL

1 Anastomosis

(1) *Hyphal Fusion*:

A study of germinating conidia shows that a considerable number of anastomoses occur soon after the germ-tubes are produced. Such anastomoses take place freely between conidia, between germ-tube and conidium, and between germ-tubes by short hyphal connections (fusion bridges), as shown in Plate 1.

For the purpose of detailed observation of such phenomena, hanging-drop cultures were made with different variants of the same strains. Conidia from 7-day-old cultures on Koji-extract agar slants were inoculated on a glass surface covered thinly with malt-extract agar (Ballg. 1°, agar 0.5 per cent, pH 5.4). They were throughly mixed, and incubated at 30°C. At intervals of 6, 12, 18, 24, and 72 hours, observation was made, and at the same time each sample was fixed in Carnoy's fluid and stained by the Feulgen[24] technique in order to clarify whether nuclear or cytoplasmic migration occurs through fusion bridges. When conidia of two strains are indistinguishable by morphological characteristics such as conidial size, structure of conidial walls, or conidial color, those of one strain were previously stained with 0.02 per cent neutral red at 30°C for 2 hours.

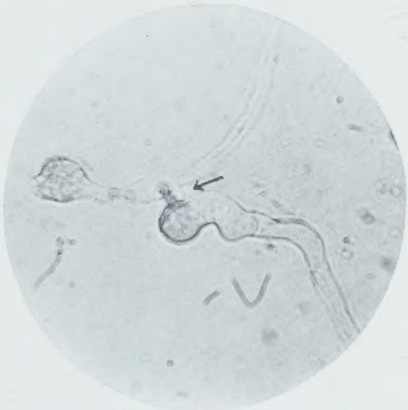
After 5–6 hours' growth of the sporelings, anastomoses were observed between branches of the same origin or between those of different origin. Conidial fusions were most abundant at distances of less than 3–10μ, and few occurred at more than 20–30μ. Thus, if two strains were grown on solid medium, starting from inoculi far apart from each other, or if single conidia of two strains were used, very few fusions occurred between them. On the other hand, if the strains were inoculated close together, much fusion occurred. Accordingly, fusion between strains seems to vary with the frequency of chance of contact. Among the 4986 fusions examined, the frequency of anastomoses between conidia, between germ-tubes, and between conidium and germ-tube, was respectively 83, 6, and 11 per cent. By conidial fusion we mean a fusion of two conidia which show only a very short or no hyphal connection. Such conidial fusions are clearly extraordinarily high, and we see that anastomoses always occur in the early stages of germination or development of hyphae. Anastomoses between mature and older hyphae, which were reported by Hansen and Smith[2], by Baker[7], and by Buxton[13], were scarcely ever observed in the course of our study. However,

Plate 1. Anastomosis—Self-fusion

Asp. oryzae K. B.

Asp. oryzae K. B.

Asp. oryzae K. B.



Asp. sojae Yellow mutant (Y_1)

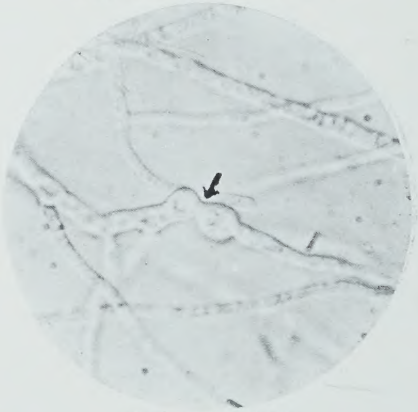
Asp. tamarii T16

Plate 1. Anastomosis—Self-fusion

Asp. wentii 32–6

Asp. carbonarius BL5–8

Asp. candidus P. O.



Asp. ustus 25–8

P. frequentans 1256

the observations of Taschdjian and Muskatblit[25] on *Trichophyton tonsurans* are in accord with ours.

The dimensions of fusion bridges pushing out from germ-tubes or hyphae of each strain were examined in mixed inoculum cultures of C-and M-type strains of *Asp. oryzae* aX. Among the 272 fusion bridges studied, 30 per cent were nearly the same, and 70 per cent were of different sizes.

Stained preparations showed that interchange of cytoplasm occurred in approximately 40 per cent of the above fusion bridges. This cytoplasmic migration was also ascertained with the help of a phase-contrast microscope. As concerns nuclear migration, more nuclei were observed in the neighbourhood of the middle part of each fusion bridge. In addition, these nuclei are seen to divide, and nuclear migrations from one hypha to another, through the bridges, were also observed. Although the details of the following problems are essential for the study of heterocaryotic systems, they remain obscure at present: 1) whether the number of nuclei of migration is constant or inconstant; 2) whether the nuclear migration is continued for considerable time through anastomoses; and 3) whether the migration of nuclei is unidirectional or reciprocal.

(2) *The Effcts of Environment on Anastomosis*:

It has been shown by many workers that anastomosis is the first step in obtaining heterocaryons. However, no work on the influence of environmental conditions on the frequency of anastomosis has been done except by Kato[26]. The establishment of constant conditions for measuring the frequency of hyphal fusions is indispensable in order to investigate the effect of the genetic constitution on anastomosis.

Kato states that a low concentration of wort, a high C/N ratio, and a low absolute amount of N-source in a synthetic medium are favorable to anastomosis. We have studied the effects of cultural conditions, such as kind of medium, concentration of medium, pH, temperature, age, etc. on anastomosis. A preliminary report[14] of this study was presented in the Journal of the Agricultural Chemical Society of Japan, 27: 638, in 1953.

Strains: Color mutants isolated by Iguchi[27] after UV treatment of *Asp. sojae* K.S.—the albino mutants (Al_1, $A1_2$, Al_3, and Al_4), the yellow mutant (Y_1), and the brown mutant (B_1).

Methods measuring frequency of anastomosis: (a) Hanging Drop Culture—The culture method is described in the previous section (1) of this series. Conidia of yellow or brown mutants could be easily distinguished from those of albino mutants both by color and structure of conidial walls. After 20–24 hours' incubation, anastomoses were observed only when there was a good mixing of conidia of both strains. Anastomoses include fusions between conidia, between germ-tubes, and between conidium and germ-tube. The frequency of anastomosis was determined by counting the number of fusions among 100 germinated conidia of each strain through examination of 50–100

$$\text{Frequency (per cent)} = \frac{\text{No. of Total Fusions}}{\text{100 Germinated Conidia of Each Strain}}$$

microscopic fields.

Triplicate preparations were studied in each test. (b) Mixed Point-Inoculum Plate Culture—The above method is very critical because the exact number of hyphal fusions is determined by microscopic observation; however, it is very laborious. In order to test a great many cultural conditions a more simple method was devised. Heavy inocula of conidia of paired strains were put on petri dishes containing various media and throughly mixed. After 5 days' incubation at 30°C, green conidial heads like the original wild type developed around the point of inoculation. When viewed under a low magnification these heads are seen to be usually composed of three different conidial chains: e.g., from a mixture of yellow and albino mutants, mixed heads with green, yellow, and albino conidial chains were observed. The frequency of occurrence of such heads was used to estimate the relative frequency of anastomosis. The experiments were carried out in quadruplicate for each pair. The determinations of frequency of occurrence of green heads were made independently by two workers and the results were very consistent. The results of the two methods used (Table 1) also agreed so exactly that

Table 1. Comparison of Anastomosis-frequency between Yellow (Y_1) and Each of Albino Mutants (Al_1, Al_2, Al_3, and Al_4) of *Asp. sojae* by Means of Hanging Drop Culture (i) and Mixed Point-Inoculum Plate Culture (ii).

Media	Combinations							
	Y_1+Al_1		Y_1+Al_2		Y_1+Al_3		Y_1+Al_4	
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
	%		%		%		%	
Rice Germ-extracts	3	+*	5	++	3	+	7	++～+++
Wort (Ballg. 1°)	6	++	12	+++	25	+++	13	++
Glucose 6 g/*l*+$NaNO_3$ 0.04 g/*l*	5	++	10	+++	20	+++	15	++
Glucose 0.2 g/*l*+$NaNO_3$ 1.6 g/*l*	2	±～+	3	±～+	4	+～++	2	+

* The relative frequency is indicated by the number of plus signs.

the second, more easy, method (b) was mainly adopted for this study.

1) The Effects of Concentrations of Various Natural Media on Anastomosis:

Koji-, malt-, rice germ-, and yeast-extracts were examined in varying concentrations of 1/100–1/1 (Table 2). The last two media were supplemented with 5 per cent glucose and 0.5 per cent peptone. Lower concentrations, about 1/10 of the normal concentration, were generally favorable to anastomosis. No differences in the frequency of anastomosis were found among these four media. This result agrees with that of Kato.

2) The Effect of Concentration of a Synthetic Medium, Czapek's Solution, on Anastomosis:

Table 2. The Effects of Concentrations of Various Natural Media on Anastomosis

Media	Concentrations	pH	Results	Media	Concentrations	pH	Results
Koji-extract	Ballg. 10°	5.4	±	Wort	Ballg. 10°	5.4	±
	5	〃	++		5	〃	++
	2	〃	+++		2	〃	+++
	1	〃	+++～++++		1	〃	+++～++++
	0.5	〃	+++		0.5	〃	+++
	0.2	〃	++		0.2	〃	++
	0.1	〃	+		0.1	〃	+
Rice Germ-extract*	1/1	5.4	+	Yeast-extract*	1/1	5.6	+
	1/2	〃	++		1/2	〃	+～++
	1/5	〃	+++		1/5	〃	++～+++
	1/10	〃	+++		1/10	〃	+++～++++
	1/20	〃	+++		1/20	〃	+++
	1/50	〃	++		1/50	〃	++
	1/100	〃	+		1/100	〃	+

* The two media were supplemented with 5 per cent glucose and 0.5 per cent peptone.

Table 3. The Effect of Concentrations of a Synthetic Medium (Czapek's Sol.) on Anastomosis

Media	C-source (Glucose g/l)	N-source ($NaNO_3$ g/l)	pH	Results
10/1 C	300	20	6.4	−
5/1 C	150	10	〃	−
2/1 C	60	4	〃	++～+++
1/1 C	30	2	〃	++～+++
C/2	15	1	〃	+++
C/5	6	0.4	〃	+++～++++
C/10	3	0.2	5.8	+++
C/20	1.5	0.1	〃	+++
C/40	0.75	0.05	〃	++～+++
C/80	0.375	0.025	5.6	+～++
C/160	0.188	0.013	5.4	+～++
C/320	0.094	0.006	〃	+～++
C/640	0.047	0.003	〃	+～++
C/1280	0.024	0.002	〃	+
Distilled-agar (Control)	0	0	〃	+

Czapek's solution was examined in concentrations of 1/1280–10/1; it was found that concentrations of 1/2 to 1/20 of the original solution gave good results for anastomosis (Table 3).

3) The Effects of Salts in Czapek's Solution:

Various solutions of substances to be tested were prepared by mixing 1/5–10/1 concentrations of two solutions composing Czapek's medium—one including glucose and $NaNO_3$ and the other including $MgSO_4$, KH_2PO_4, KCl, and $FeSO_4$. No significant effect on anastomosis was seen in media made up with and without salts.

Recently Humphreys Jones[28] stated that varying K and NO_3 ions produced no effects on hyphal fusions in *Fusarium culmorum*.

4) The Effects of Relative Concentrations of C- and N-sources on Anastomosis:

These examinations were made using glucose as a C-source and $NaNO_3$ as a N-source, and by varying of amounts of C- and N-sources as shown in Table 4.

The maximum C/N ratios of the A and B groups were 3840:1 and 1:16 respectively, and that of the D group is 1/1. The A group was kept constant in N-source (0.1g/1), and the B group was constant in C-source (6g/1). As is clearly shown for both groups in the Table, the higher C/N ratio generally favors anastomosis, especially when the N-source in the

Table 4. The Effects of Relative Concentrations of C–(Glucose) and N–($NaNO_3$) Sources on Anastomosis.

Media	A (with $NaNO_3$ 0.1 g/*l*)		B (with Glucose 6 g/*l*)		D		
(No.)	Glucose (g/*l*)	Results	$NaNO_3$ (g/*l*)	Results	Glucose (g/*l*)	$NaNO_3$ (g/*l*)	Results
1	384	+	0.002	++~+++			
2	192	++~+++	0.004	++~+++			
3	144	+++	0.006	++~+++			
4	96	+++	0.007	++~+++	96	96	±
5	48	+++	0.013	++++	48	48	+
6	24	+++	0.025	++++	24	24	++
7	6	+++	0.1	+++	6	6	+++
8	1.5	+++	0.4	+++	1.5	1.5	++
9	1.0	++~+++	0.6	+++	1.0	1.0	++
10	0.4	++	1.5	+++	0.4	0.4	++
11	0.2	++	3	+++	0.2	0.2	++
12	0.1	++	6	+++	0.1	0.1	++
13	0.05	++	12	++	0.05	0.05	++
14	0.01	++	24	++			
15	0.005	++	48	+			
16	0.001	++	96	±			

medium is lower than in the original Czapek's solution. A fairly low concentration of N-source, 48g/l (0.56 mol), inhibited anastomosis. On the other hand, no such inhibition was shown by the C-source, even in a higher concentration, 384g/l (2 mol).

5) The Effect of Osmotic Pressure on Anastomosis:

Table 5. The Effect of Osmotic Pressure on Anastomosis.

Media (No.)	Concentrations (mol)	NaCl	Glucose	Lactose
1	2	±～+	+	++～+++
2	1	+	++～+++	++～+++
3	0.5	++～+++	++～+++	+++
4	0.3	++	++	+++
5	0.2	++～+++	+++	+++
6	0.1	+++	++～+++	+++
7	0.05	++～+++	++～+++	++～+++
8	0.02	++～+++	++～+++	++
9	0.01	++～+++	++～+++	+～++
10	0.005	++	++	+

From the results of the above experiments, it is seen that the frequency of anastomosis can be influenced only by the concentration of the medium. Therefore the effect of osmotic pressure was examined with varying concentrations (0.005–2mol) of NaCl, glucose, and lactose (Table 5).

The optimum concentrations of NaCl, glucose, and lactose were 0.1, 0.2, and 0.1–0.5 mol, respectively. Higher or lower osmotic pressures was found to be less favorable. The rather high optimal concentration of lactose seems to be due to a low permeability of the cell-wall of *Aspergillus*.

6) The Effect of pH of Medium on Anastomosis:

Basal media containing 1 per cent glucose plus 0.1 per cent peptone, or 1 per cent glucose plus 0.1 per cent $NaNO_3$, were used. The reaction of the media was changed by adding phosphate buffer[29] so that values between pH 2.2 and 8.8 could be examined.

The range of the optimal pH zone was rather narrow, from 5.4 to 6.0 (Table 6). The ability to anastomose decreased rapidly at low pH's and slowly at the higher side of the optimal zone. Hyphal fusions were completely inhibited below pH 4 and at 8.8. Humphreys Jones[28] also showed that the pH of the medium affected hyphal fusions in *Fusarium*.

7) The Effect of Age of Culture on Anastomosis:

Stock cultures preserved on Koji-extract agar slants for 1/4, 1, 3, 6, and 12 months were used. Between one week and three months age did not significantly influence anastomosis; from 3 to 6 months a slight decrease was observed, and an incubation period longer than 6 months seemed to decrease the frequency a little more.

8) The Effect of Temperature on Anastomosis:

This variable was studied by combining the yellow (Y_1) and albino (Al_4) mutants in hanging drop cultures at temperatures of 20°, 25°, 30°, and

Table 6. The Effect of pH on Anastomosis.

*Media P-series No.	pH	Results	*Media C-series No.	pH	Results
1 (Control)	5.8	###	1 (Control)	5.8	###
2	2.2	±	2	2.2	±
3	3.4	±			
4	4.0	±	4	4.0	±
5	4.6	##			
6	5.4	##～###	6	5.4	##～###
7	6.0	##～###	7	6.0	##～###
8	6.4	##	8	6.4	##
9	6.6	##			
10	7.0	+～##	10	7.0	+
11	7.4	+～##			
12	7.6	+			
13	8.0	+	13	8.0	+
14	8.8	±	14	8.8	±

* In the P-series, glucose 1% plus peptone 0.1% and in the C-series, glucose 1% plus $NaNO_3$ 0.1% were used as basal media. The range of pH was changed by adding phosphate-buffer to the basal media.

37°C. The optimum temperature for anastomosis lies between 25° and 30°C; this is somewhat lower than that for the optimum growth of the Koji-molds (Table 7).

Table 7. The Effect of Temperature on Anastomosis.

Media	Temperature (°C)			
	20	25	30	37
	%	%	%	%
Wort (Ballg. 1°)	3	25	19	8
〃 (〃 8°)	1	3	2	0
Koji-extract(〃 1°)	0	18	17	1
〃 (〃 8°)	1	4	2	1
Glucose 6 g/l+$NaNO_3$ 0.4 g/l	3	15	15	1
Glucose 1%+Peptone 0.1% (pH 5.8)	0	17	14	2

The effect of environment on anastomosis of Koji-molds has been studied by hanging drop culture and mixed point-inoculum plate culture of color mutants of *Asp. sojae*. The important factors which influence hyphal fusion are the concentration and the pH of the media. Optimal conditions for anastomosis lie between 1/5 and 1/20 of the original concentration of media, both natural and synthetic, and the optimal pH-range is 5.4 to 6.0. It may be suggested from experiments of this series that the frequency of anastomosis seems to be increased by poor nutritional conditions. In this connection, Buxton[13] recently stated that anastomosis in *Fusarium oxysporum* appeared to be

more frequent when conidia germinated in water agar. Sermonti[80] also used limiting media for the production of heterocaryons in *Penicillium*. The media were prepared by adding the requirements of the two strains to minimal media in limiting concentrations. The concentrations were less than those needed for optimum growth, and allowed only stunted growth of the original strains. Although he did not observe hyphal fusions directly on such media, he obtained positive results in the formation of heterocaryons. These reports are exactly in accordance with Kato and the authors.

The C/N ratio is also important, and we observe that higher ratios increase anastomosis.

Table 8. The Effect of Conidial Staining (0.02% Neutral Red) on Germination and Self-fusion Frequency of Each Strain of *Asp. oryzae* and Interstrain Fusion (Tester: *Asp. oryzae* K.B.).

Strains		Colony Types	Germination Ratio (%)		Self-fusion of Each Strain (%)		Inter-strain Fusion (%)	Self-fusion of *A. oryzae* K.B. (%)
			No Staining	Staining	No Staining	Staining		
	K. B.	C	100	94	31	29		
	S4-18	C	87	92	33	31	16	28
A. oryzae s. str.	020-3	C	92	90	38	43	17	29
	S1-2	MC	100	76	29	29	16	30
	S4-15	MC	99	89	22	25	15	31
	S7	MR	94	85	21	19	20	31
	B	M	99	92	22	25	17	25
	SH15-3	C	90	93	31	30	16	33
A. oryzae var. *micro-sporus*	S3-4	C	96	88	33	34	17	31
	SH 22	MC	98	90	30	25	18	33
	G	M	97	88	25	26	19	37
	F	M	92	80	21	23	15	26
	SH-34	C	99	93	24	31	17	36
	S2-3	MC	96	73	31	36	17	32
A. oryzae var. *magnasporus*	A5-3	MC	100	85	29	32	19	36
	H1-1	M	96	94	22	24	16	30
	A5-7	M	100	96	28	28	21	33
	SH 31	M	99	91	26	24	16	34
A. oryzae var. *globosus.*	M2-1	C	96	73	30	28	15	36
	S6-6	M	99	94	33	30	16	30
A. oryzae var. *fulvus*	K	C	100	99	37	40	12	35
Average			97	88	28	29	17	31

(3) *The Influence of Genetic Conditions on Anastomosis (On the Occurrence of Anastomosis between Species and between Genera of Molds)*:

It has already been recognized by many workers that heterocaryons are produced through anastomosis between mutants or variants of a strain. However, there is as yet very little information about anastomosis between different species with two exceptions. Kato[26] reported that anastomosis between *Penicillium notatum* 1249 and *P. chrysogenum* Q176 took place only once in 40 experiments, and the heterocayon showed no further development. Hansen[31] stated that heterocayotic crosses between different well-characterized species (*Botrytis allili* and *B. vicini*) were obtained, yielding progeny resembling both parents or progeny decidedly different from either parent.

An attempt[15] to investigate the occurrence and frequencies of anastomosis between different strains, between different species, and between different genera has been made using *Asp. oryzae* K. B. and a yellow mutant stain of *Asp. sojae* (Y_1) as test organisms. In the course of this experiment we were interested in observing the difference in compatibility among various fungi. Further studies to elucidate the possibility of the formation of heterocaryons between different species are the subject of continuing research.

Strains used and combinations of strains: (a) Between different strains of Koji-molds (Table 8)—*Asp. oryzae s. str.* (6 strains), *Asp. oryzae var. microsporus* (5), *Asp. oryzae var. magnasporus* (6), *Asp. oryzae var. globosus* (2), and *Asp. oryzae var. fulvus* (1). These were isolated and classified by Sakaguchi and Yamada[33] and have been fully described in previous sections (I[33] and IV[34]) of this series. (b) Between different species (Table 9)—Aspergilli (17 species). These species are typical strains of groups described by Thom and Raper[35] in their *Manual of the Aspergilli*. (c) Between different genera (Table 10)—Penicillia including the Monoverticillata (5 species), the Biverticillata asymmetrica (7), and the Biverticillata symmetrica (7). These species were obtained through the courtesy of S. Abe. In addition, *Monascus* (5 strains) was examined. (d) Other strains—*Monilia* (2 strains), *Mucor* (4 species), and *Rhizopus* (4 species). All strains mentioned above were combined with *Asp. oryzae* K. B. which is a test strain. For analysis of anastomosis between species, the yellow mutant strain (Y_1) of *Asp. sojae* K. S. was used as an additional test strain.

Method for estimating the frequency of anastomosis: The frequency of anastomosis was measured by the hanging drop culture described previously, with rigid control of the culture conditions. When conidia of two strains were indistinguishable, vital staining of the conidia of one was made with 0.02 per cent aqueous neutral red at 30°C for 2 hours. Pinkish granules were clearly observed inside such conidia or new hyphae. Stained conidia showed an average decrease of 9 per cent in frequency of germination, but there was no effect of the strain on normal development of the germ-tube or the frequency of self-fusion (Table 8).

Table 9. Interspecific Fusion.

Strains		(Tester: *A. oryzae* K.B.)			(Tester: *A. sojae* Yellow Mutant (Y_1)		
		Self-fusion of Each Strain (%)	Inter-specific Fusion (%)	Self-fusion of *A. oryzae* K.B. (%)	Self-fusion of Each Strain (%)	Inter-specific Fusion (%)	Self-fusion of *A. soiae* Yellow Mutant (Y_1) (%)
A. oryzae	K.B.				35	22	35
A. sojae	SH10–3	20	11	36			
A. sojae Albino Mutant	$A1_4$				32	22	32
A. tamarii	T16	31	14	33	32	11	33
	23–12	29	13	30			
A. ochraceus	24–6	16	11	31	20	11	28
	24–4	14	10	32			
A. wentii	32–6	18	8	31	21	10	30
	23–2	10	7	30			
A. niger	BL5–1	18	13	32	18	12	26
A. carbonarius	BL5–8	16	11	26	23	13	27
A. usamii	R-0635	23	14	30	24	13	32
A. awamori	RL5–9	14	8	32	15	9	32
A. candidus	P0	10	3	24	12	2	25
	B.F.	15	4	27			
A. terreus	26–13	13	2(1)	23	16	1(2)	26
A. versicolor	A130	7	(3)	27	10	(3)	27
A. sydowi	25–4	5	2(1)	25	5	2	24
A. flavipes	A10–2	7	(3)	23	6	(5)	23
A. ustus	A10–4	4	(3)	26	6	(2)	26
	A31	2	(3)	28			
A. nidulans	A477	1(6)*	(2)	22	4	(1)	25
A. fumigatus	A44	2	(2)	22	4	(2)	28
	A24	3	(2)	24			
A. glaucus	A1–8	5	3	25	6	3	27
A. clavatus	A1–1	5	(1)	29	4	(1)	28

* ( ): The occurrence of fusion is uncertain.

1) Between Different Strains of Koji-molds:

The frequency of fusions between *Asp. oryzae* K. B. and 20 different strains of Koji-molds was from 15 to 20 per cent. No significant differences according to the kinds of strains were found. Only *Asp. oryzae var. fulvus* K showed a somewhat lower (12 per cent) frequency. Lindegren and Andrews[36] reported different frequencies of fusions in different combinations of strains of *P. notatum*. However, this was not observed in our experi-

Table 10. Intergeneric Fusion (Tester: *Asp. oryzae* K.B.).

Strains			Self-fusion of Each Strain (%)	Intergeneric Fusion (%)	Self-fusion of *A. oryzae* K.B. (%)
Monoverticillata	*P. frequentans*	1256	23	3	27
	P. multicolor	1051	12	5	30
	P. implicatum	807	2(8)	(2)	29
	P. trzebiuski	631	23	2	32
	〃	251	20	3(4)	39
	P. spinulosum	668	6	(2)	32
Biverticillata asymmetrica	*P. citrinum*	1131	(4)	(1)	26
	P. notatum	578	(6)	(2)	40
	P. chrysogenum	Q176	0	0	36
	P. oxalicum	1180	2(3)	(1)	30
	P. roqueforti	731	10	(1)	33
	P. brevi-compactum	1235	1(2)	0	34
	P. claviforme	1162	20	(2)	37
Biverticillata symmetrica	*P. purpurogenum*	722	40	(1)	32
	P. concavo-rugulosum	683	10	(2)	33
	P. aculeatum var. apiculatum	1207	1(2)	(1)	28
	P. herquei	953	18	2	30
	P. verrculosum	1114	3(1)	(1)	35
	〃	961	5(1)	1(1)	31
	Monascus	Ma 3-1	6	0	26
		Ma 1-3	0	0	28
		Ma 1-4	1	0	30
		Ma 1-9	0	0	29
		Ma 2-1	(10)	0	30

ments; the differences between strains were not significant. Self-fusion of each strain is generally high (maximum 38 per cent, minimum 21 per cent).

2) Between Different Species:

On the basis of the frequency of interspecific fusions with the test organisms, 17 other species of *Aspergillus* could be divided into 2 groups. Group A: those which have high frequencies (ca. 10 per cent)—*Asp. tamarii*, *Asp. ochraceus*, *Asp. wentii*, *Asp. niger*, *Asp. carbonarius*, *Asp. usamii*, and *Asp. awamori*. Group B: those which have low frequencies (1–2 per cent)—*Asp. candidus*, *Asp. terreus*, *Asp. versicolor*, *Asp. sydowi*, *Asp. flavipes*, *Asp. ustus*, *Asp. nidulans*, *Asp. fumigatus*, *Asp. glaucus*, and *Asp. clavatus*. Strains of the first group are also generally high in frequency of self-fusions, while most strains of the second group are much lower. If we suppose that

species showing fusions in high frequency are closely related, the species belonging to group A might be regarded as in closer relationships to the Koji-molds (*Asp. oryzae* and *Asp. sojae*). It is very interesting that these relationships in the frequency of anastomosis are correlated with the natural relation among *Aspergillus* species given by Thom and Raper[35]. There was almost no difference in frequency of fusion whether the strains were tested with *Asp. oryzae* K. B. or with the yellow mutant of *Asp. sojae* (Y_1) (Table 9).

3) Between Different Genera:

Intergeneric fusions between *Asp. oryzae* K. B. and *Penicillium* or *Monascus* are shown in Table 10. Among the Penicillia anastomosis with the test organism took place only in species which belong to the Monoverticillata and in species of the Biverticillata whose frequency of self-fusion is extremely high. In the species belonging to *Rhizopus* and *Mucor* anastomosis with *Asp. oryzae* K. B. did not occur. Self-fusion in these strains was very rare.

2. Production of Heterocaryons.

Our previous work indicates that anastomosis occurs not only between mutants or between strains of Koji-molds, but also between species of *Aspergillus* and between genera (*Asp. oryzae* K.B. and some members of the Penicillia). It remains, however, for the authors to investigate much more interesting problems: 1) whether or not those anastomoses immediately result in heterocaryotic conditions, and 2) whether if heterocaryons are produced, their phenotype or stability would be altered. The choice of genetic markers for these studies is most important; size of conidia, color of conidia, and nutritional requirements were adopted by the authors as they allow certain identification. Heterocaryons have been achieved by ultraviolet treatment of homocaryotic strains without hyphal fusion. This will also be described below.

(1) *Size of Conidia*:

In previous work[34] the authors showed ample evidence that the inconstant, hetero-type strains have generally large, multinucleate (8–20 or more) conidia; on the other hand, the constant, homo-type strains form small, binucleate conidia (about 95 per cent contain two nuclei). An attempt[19–20] to produce hetero-type strains (X) was made by mixing cultures of homo-type segregants.

Conidia of conidial (C)- and mycelial (M)-type strains, which were obtained from *Asp. oryzae* aX by pedigree culture, were mixed on wort-agar slants (Ballg. 1°, pH 5.4). After 7 days' incubation the cultures were analyzed by ordinary single-spore isolation technique. At the first transfer intermediate type colonies(X) were obtained in addition to the C- and M-types. As a check, mixed cultures of combinations of one type (C+C and

M+M) were examined by single-spore culture. The first gave all C-type and the second all M-type. Further tests for the X-type strains obtained were carried out by pedigree cultures. The X-type strains always segregated out C-, M-, and X-types in each successive transfer, as shown in Table 11. It is therefore assumed from the behavior of the progeny that heterocaryotic strains having nuclei of both parents were synthesized by mixed culture, probably through anastomoses of germ-tubes and subsequent migration of nuclei.

Table 11. Pedigree Culture of Hetero-type (X) Obtained from Mixed Culture of Homo-type Segregants (C and M) of *Asp. oryzae* aX.

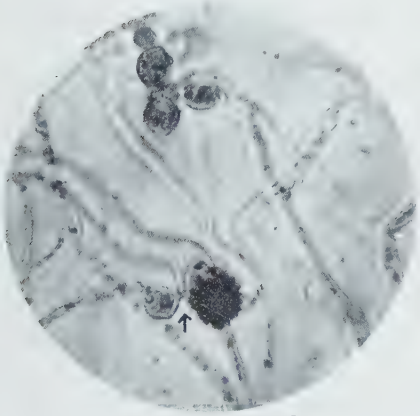
(Transfer)

| | | |

Plate 2. Anastomosis—Interspecific or Intergeneric Fusion
left row: Tester—*Asp. oryzae* K. B. right row: Tester—*Asp. sojae* Yellow mutant (Y_1)

Asp. wentii 32-6

Asp. glaucus A1-8



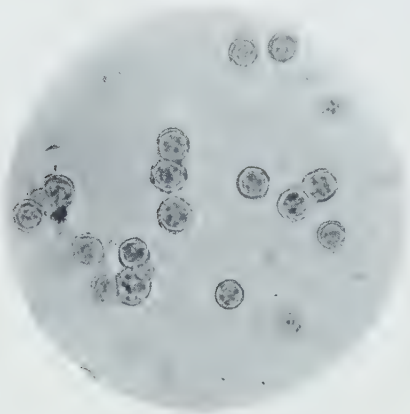
Asp. carbonarius BL5-8

Asp. carbonarius BL5-8

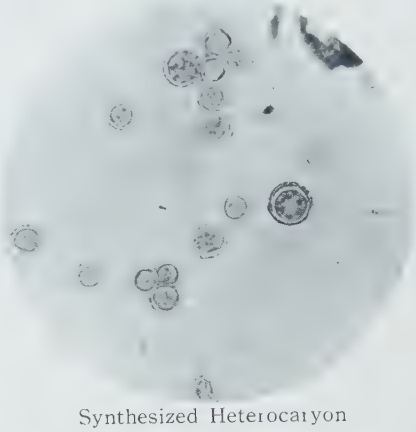
P. aculeatum var. 1207

P. frequentans 1256

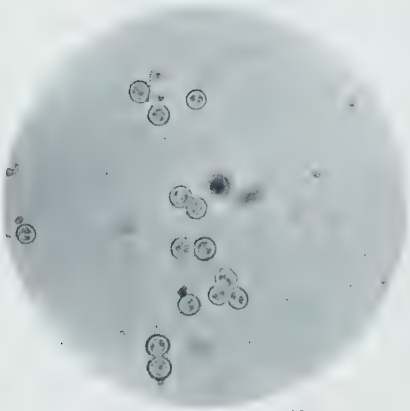
Plate 3. Conidia of the Original Parent Strain, its Homo-type Segregants, and a Synthesized Heterocaryon of *Asp. oryzae var. magnasporus* SH8-4



Original Parent Strain

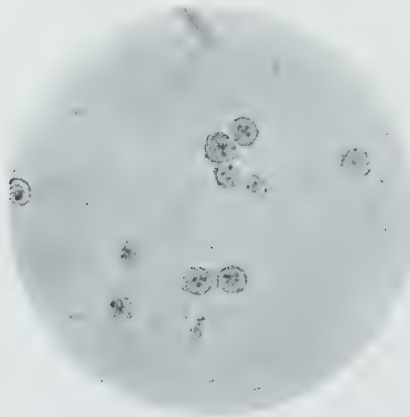


Synthesized Heterocaryon

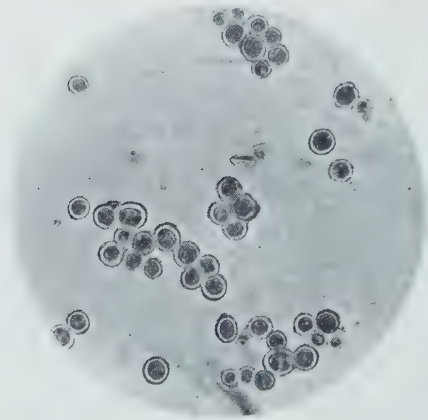


Homo-type Segregant (C-type)

Homo-type Segregant (C_2 mm)



Homo-type Segregant (M-type)



Homo-type Segregant (C_5 mm)

than those of that original parent strains: C-type (3.5–5.5μ), M-type (4–6μ), C_2mm-type (3.5–5.5μ), and C_5mm-type (5–7μ) (Plate 3). The large conidial strains (X-type) similar to the original parent were synthesized by mixed culture of those homo-type segregants as shown in Fig. 1, and each component homo-type could again be segregated out by subsequent single-spore analysis.

From the above facts it is possible to use the size of conidia as an aid to isolate heterocaryons. This could be done by selecting larger conidia from mixed cultures of homo-type segregants. Additional evidence, which will be described below, is the detection of green strains produced by mixing different color mutant or prototrophic heterocaryons obtained by crossing nutritional mutants.

(2) *Color Markers of Conidia*:

The production of heterocaryons similar to wild type from a mixed culture of two mutants which differ only by a color marker, has already been confirmed by many workers (Gossop, Yuill, and Yuill[37]; Beadle and Coonradt[16]; Pontecorvo and Gemmell[9]; Raper[38]; and others). For example, Gossop *et al* observed almost black heads from a mixed culture of brown- and cinnamon-colored mutants of *Asp. niger*. The black type yielded some cinnamon, some brown, and some mixed colonies; in which again cinnamon, brown, and black heads could be identified. Such phenomena were interpreted as representing a heterocaryotic fructification which had developed as a result of hyphal anastomoses with subsequent nuclear intermixture of the two mutant types.

Combinations between mutants or between a yellow mutant of *Asp. sojae* and other species were investigated by mixed point-inoculum plate culture.

Strains used and combinations of strains:

Mutants—albino (Al_1, Al_2, Al_3, Al_4, Al_5, Al_6, and Al_7), yellow (Y_1 and Y_2), and brown (B_1 and B_2). Different species of *Aspergillus* and *Penicillium*. Almost all strains used for the study of genetic condition on anastomosis were again used in this study.

The method used is the same as described in the previous section. The conidia of two strains were point-inoculated on plates of wort agar (Ballg. 1°) or of 1 per cent glucose and 0.1 per cent peptone agar. After 5 days' incubation, the capacity to form green, heterocaryotic heads and the degree of their development were examined.

1) Between Mutants:

Among all combinations of color mutants of *Asp. sojae*, green heads were produced in the following combinations: a) between yellow and all albino or brown mutants, b) between brown and all albino mutants, and c) between Al_4, induced by ultraviolet light, and Al_6 or Al_7, induced by nitrogen mustard. The frequency of production of green heads in b and c was much

Table 12. Comparison of Frequencies of Green Conidial Heads Produced by Various Pairs of Color Mutants of *Asp. sojae*.

Mutants	Degree of Coloration When Mix-cultured with										
	Y_1	Y_2	B_1	B_2	Al_1	Al_2	Al_3	Al_4	Al_5	Al_6	Al_7
Y_1*	−										
Y_2	−	−									
B_1	###	##	−								
B_2	##～###	##	−	−							
Al_1	##	##～###	±	±	−						
Al_2	##～###	#～##	+～#	+～#	−	−					
Al_3	###	###	#	#	−	−	−				
Al_4	###	###	#	#	−	−	−	−			
Al_5	###	##～###	±	±	−	−	−	−	−		
Al_6	##～###	##～###	+	+	−	−	−	+	−	−	
Al_7	###	###	+～#	+～#	−	−	−	+	−	−	−

* Yellow (Y_1 and Y_2), brown (B_1 and B_2), and albino (Al_1, Al_2, Al_3, Al_4, Al_5, Al_6, and Al_7) mutants were mixed by point-inoculum plate cultures between various pairs. These strains were produced by UV (Al_1, Al_2, Al_3, Al_4, Y_1, and B_1), X-rays (Al_5, Y_2, and B_2) and nitrogen mustard (Al_6 and Al_7).

o wer than in a (Table 12).

2) Between Y_1 and Other Strains:

In mixed cultures of a yellow mutant (Y_1) with other species of *Aspergillus* or *Penicillium* whose conidial heads could be distinguished from *Asp. sojae*, green conidia characteristic of *As. sojae*, green conidia were also formed. The frequency of the formation nearly paralleled the frequency of anastomosis as shown in Table 13.

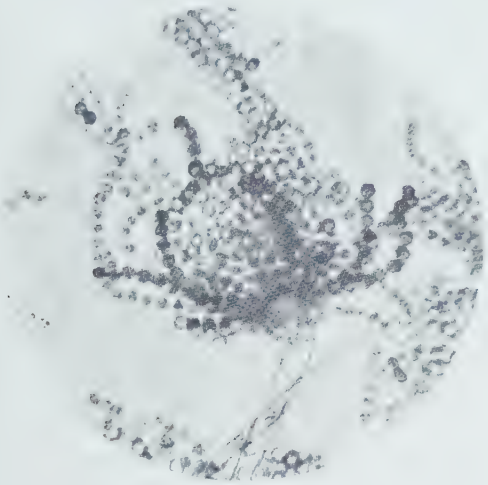
3) Y_1 Tested on Culture-filtrates:

The above results show that green heads are produced in mixed cultures of Y_1 and other strains, as well as between mutants. The facts therefore indicate the possibility of the production of green heads by some supposed common substance existing in these strains. To test this suggestion, various culture filtrates were prepared from 5-day-old cultures of Al_4, *Asp. ochraceus* 24-6, *Asp. niger* BL5-1, *Asp. usamii* R-0635, and *P. frequentans* 1256 on wort (Ballg. 1°) by filtration with a Seitz filter. A yellow mutant (Y_1) was allowed to grow on agar containing one of the culture filtrates at 30°C for 5 days. Yellowish green conidia were produced in all cases. Other conditions for green conidia formation have been further studied by Takagi[39].

4) Between Yellowish Brown-and White-spored Strains of *Aspergillus*:

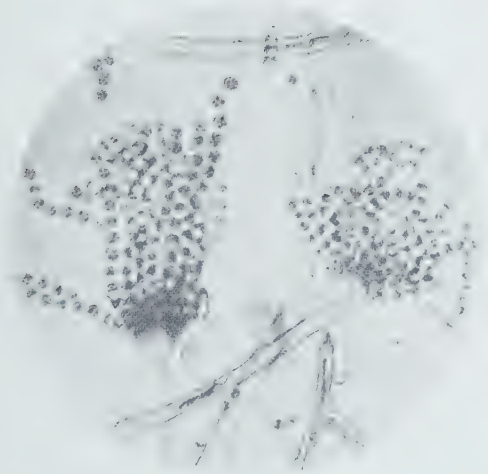
In all possible combinations of a yellowish brown *Aspergillus* (*Asp. tamarii* T16, A119, *Asp. wentii* 32-6, 23-2, *Asp. ochraceus* 24-6, and *Asp.*

Plate 4. Conidial Heads Produced from Mixed Culture of a Yellow Mutant (Y_1) and an Albino Mutant (Al_4)

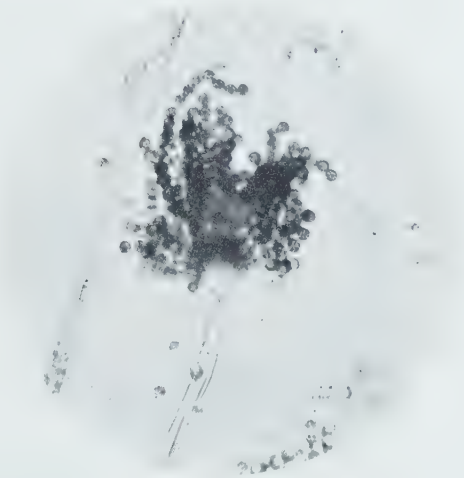


Green and Yellow or Albino Conidial Chains

Green and Yellow or Albino Conidial Chains



Yellow Conidial Chains (Y_1)



Green Conidial Chains (Wild Type)

Table 13. Combinations of Yellow Mutant (Y_1) of *Asp. sojae* with Strains of *Aspergillus* or *Penicillium* and the Production of Green Conidial Heads.

Combinations		Hanging-drop Cultures		Point Mixed Plate Cultures (Degree of Coloration)	
		Germination Ratio (%)	Anastomosis Frequency (%)	on Wort Agar (Ballg. 1°)	on Glucose 1% + Peptone 0.1% Agar
A. sojae Albino Mutant	Al_4	98	22	###	###
A. sojae Brown Mutant	B_1	98	20	###	###
A. oryzae Albino Variant	S2–3	96	15	##～###	###
A. oryzae Albino Mutant	S16A	92	8	###	###～###
A. tamarii	T16	97	11	##	###～###
A. ochraceus	24–6	90	11	###	###
A. wentii	32–6	92	10	###	###
A. niger	BL5–1	95	12	###	##～###
A. carbonarius	BL5–8	95	13	###	###
A. usamii	R–0635	96	13	###	###
A. awamori	BL5–9	68	9	##	##
A. candidus	PO	35	2	##～###	###
A. terreus	26–13	44	1(2)	+	+
A. versicolor	A130	21	(3)	+	+～##
A. sydowi	25–4	76	2	+	+～##
A. flavipes	A 1–2	95	(5)	+	+
A. ustus	A10–4	94	(2)	##	##
A. nidulans	A477	94	(1)	+	+
A. fumigatus	A44	92	(2)	##	##
A. glaucus	A1–8	30	3	+	###
A. clavatus	A1–1	88	(1)	+	+
P. frequentans	1256	98	3	+	###
P. multicolor	1051	98	4	+～##	###
P. herquei	953	98	2	+	###
A. sojae Yellow Mutant	Y_1	98	30	−	+

terreus 26–13) and a *white Aspergillus* (*Asp. candidus* PO, and albino mutant S16A of *Asp. oryzae* aX, and Al_1 or Al_4), no green coloration was found.

Among green heads developed after mixed culture of two mutant, e. g. yellow (Y_1) and albino (Al_4), two kinds are recognized: one is composed of homogeneous green conidial chains, and the other of mixed conidial chains showing all three colors (yellow, albino, and green) in varying proportions (Plate 4). These seem exactly like those described by Gossop and the Yuills[37] in *Asp. tamarii*, by Pontecorvo in *Asp. nidulans*[10][40], and by Raper

and Fennell[12] in *Asp. fonsecaeus*. Adopting the terminology introduced by Pontecorvo, the mixed heads would be designated as "autonomous" as pigmentation is determined by the nucleus segregated in each conidium.

Upon single-spore analysis of green conidia from mixed heads, about fifty per cent of the conidia gave rise to mixed colonies containing green heads and the conidial structures of each mutant. It is therefore recognized that those green conidia are with certainty heterocaryons which arose as a result of hyphal anastomoses and nuclear intermixture. However, most of the rest of the conidia gave rise to pure colonies like the original yellow mutants. Green conidia, derived from the mixed culture of a yellow mutant (Y_1) with other strains, or green conidia which were obtained by inoculating Y_1 on media with culture filtrate, yielded only pure populations of the original yellow mutant in subsequent generations. In these cases it seems that the green conidia were produced only by the effect of the cytoplasm, through anastomoses or by the temporary action of the filtrate. It is accordingly concluded that proof of heterocaryosis, using only the color of the conidia as a criterion, is rather uncertain without genetic confirmation.

(3) *Nutritional Markers*:

Attempts to force heterocaryosis by using two different biochemical mutants have been made by many workers (Ryan[41]; Giles[42]; Gross[43]; Garnjobst[44] etc.) in *Neurospora* since the pioneer work of Beadle and Coonradt[6]. In *Aspergillus* and *Penicillium* similar experiments were initiated by Pontecorvo[40][45] *et al* and Bonner[46]. Our first experiments with *Aspergillus*[17] were carried out by examining the capacity of prototrophic heterocaryons, which grow normally on M.M., like the wild type, in various combinations of single auxotrophs. The second tests[18] were performed with double auxotrophs in order to avoid possible errors of reverse mutation or contamination by other strains, which may occur with single mutants.

Strains: Single auxotrophs—8 amino acid-requiring strains among 37 single auxotrophs of *Asp. sojae* K.S. obtained by Iguchi[47-48] after treatment with various mutagens (UV, X-ray, and nitrogen mustard); lysineless—X-208 (1St *lys*), X-809 (2St *lys*), X-747 (6St *lys*), and M-535 (20C *lys*); histidineless—X-1125 (3MCR *his*) and X-398 (10C *his*); leucineless—X-793 (14C *leu*) and U-191 (17C *leu*). Double auxotrophs—9 double mutants[18][49] among 48 mutants obtained by UV irradiation of leucineless (14C *leu*) and histidineless (10C *his*). These mutants proved to be comparatively stable in colony and nutritional characters, as described in a previous section[49] of this series. Six of these strains were derived from 14C *leu*; L326 (C *leu meth*|*homo*), L467 (St *leu meth*), L490 (C *leu arg*|*cit*|*or*), L520 (C *leu lys*), L387 (*b leu ad*|*hyp*), and L415 (y *leu nic*). The other 3 strains were obtained from 10C *his*; H123 (C *his arg*|*cit*|*or*), H215 (C *his arg*), and H249 (CR *his meth*|*homo*).

Combinations (Tables 14 and 15): With single mutants heterocaryon tests were carried out in all possible combinations of (*lys*+*leu*), (*lys*+*his*), and

Table 14. The Occurrence of Prototrophic Heterocaryons between Certain Single Auxotrophic Mutants of *Asp. sojae*.

Combinations (*lys*+*leu*)	* S. F.	** P. F. (%)	Colony Types of Hetero-caryon	Combinations (*lys*+*his*)	S. F.	P. F. (%)	Colony Types of Hetero-caryon	Combinations (*his*+*leu*)	S. F.	P. F. (%)	Colony Types of Hetero-caryon
6St *lys*+14C *leu*	1:2	4.8	Cm	6St *lys*+10C *his*	1:1	2.7	Cm	10C *his*+14C *leu*	1:5	2.4	C
6St *lys*+17C *leu*	1:5	0.4	Cm	6St *lys*+3MCR *his*	1.5:1	0.3	MC	10C *his*+17C *leu*	1:6	0.5	C
1St *lys*+14C *leu*	1:3	0.2	Cm	1St *lys*+10C *his*	1:4	2.2	Cm	3MCR *his*+14C *leu*	1:7	0.02	C
1St *lys*+17C *leu*	1:2	0.5	Cm	1St *lys*+3MCR *his*	2:1	0.5	MC	3MCR *his*+17C *leu*	1:4	0.06	C
2St *lys*+14C *leu*	1:3	0.3	Cm	2St *lys*+10C *his*	1:1.7	1.4	Cm				
2St *lys*+17C *leu*	1:3	0.5	Cm	2St *lys*+3MCR *his*	1.5:1	0.4	MC				
20C *lys*+14C *leu*	1:4	0.3	C	20C *lys*+10C *his*	1:1.5	1.6	Cm				
20C *lys*+17C *leu*	1:3	0.6	C	20C *lys*+3MCR *his*	1:1	0.8	MC				

* S. F.: Frequency of conidia of each strain as determined by plating.

** P. F.: Frequency of prototrophic heterocaryon.

The conidia of two strains were mixed on a Koji-extract agar plate (Ballg. 1°) and after incubation (30°C, for 7 days), were plated on minimal medium (Czapek's agar). The number of prototrophic heterocaryons, able to grow on minimal medium, was calculated as a functions of the number of plated conidia.

Table 15. The Prototrophic Heterocaryons Occurring in Mixtures of Double Auxotrophic Mutants of *Asp. sojae*.

Strains Used for Combinations	Test Strains								
	H123 C *his arg/cit/or*			H215 C *his arg*			H249 CR *his meth/homo*		
	S. F.*	P. F.* (%)	Colony Type of Het.	S. F.	P. F. (%)	Colony Type of Het.	S. F.	P. F. (%)	Colony Type of Het.
L326 C *leu meth/homo*	1:1	8.9	C	1:1	2.1	C	1:2	0.7	C
L467 St *leu meth*	2:1	2.8	C	1:1	1.6	C	2:1	4.7	C
L490 C *leu arg/cit/or*	1:1	0.8	C	1:1	4.5	C	1:7	1.6	C
				3:1	2.5				
				4:1	5.1				
L520 C *leu lys*	1:2.5	3.4	C	1:2	1.3	C	1:4.5	0.5	C
L387 b *leu ad/hyp*	1:3	7.1	C	1.5:1	0.2	C	1:1	2.3	C
				5:1	0.3				
L415 y *leu nic*	1:2	7.0	C	1:2	0.5	C	1:3	5.2	C
				5:1	0.3				

* See Table 14.

(*his*+*leu*). With double mutants, tests were made between the 3 strains originating from 10C *his* and the 6 auxotrophs from 14C *leu*.

Conidia of two strains of 7-day-old cultures grown on Koji-extract agar slants were heavily inoculated at the same point on Koji-extract agar plate (Ballg. 1°), and mixed throughly. The plates were kept at 30°C for 5–7 days. Conidial suspensions prepared from colonies developing after mixed culture were plated onto minimal medium and at the same time onto Koji-extract agar at an appropriate dilutions. On the former medium only prototrophic heterocaryons developed; on the latter, three colony types, including each component strain and the heterocaryon, appeared (Plates 5 and 6). By counting the colonies developing on the latter medium it is possible to estimate the frequency of heterocaryotic spores. There is little difference in this frequency between single mutants (0.4–4.8 per cent) and double mutants (0.5–8.9 per cent). Only two combinations (3MCR *his*+14C *leu* and 3MCR *his*+17C *leu*) among the single mutants gave much lower frequencies, i.e. for the former 0.02 per cent and for the latter 0.06 per cent.

In the course of this study the effect of the concentration of natural media on the prototrophic heterocaryon ratio was examined by using Koji- and malt-extract (Ballg. 1° and 10°). The higher ratios of heterocaryon formation were obtained on those media favoring anastomoses, that is, the lower concentrations of natural media (Table 16). The ability to grow on M.M. is not conclusive evidence of heterocaryosis, because growth on M.M. can occur due to symbiosis without fusion of hyphae, and also due to reverse mutation. The proof of heterocaryosis is given by the demonstra-

Table 16. The Effects of Concentrations of Natural Media on Frequency of Prototrophic Heterocaryons (P. F.).

Media		Combinations					
		6St *lys*+14C *leu*		6St *lys*+10C *his*		10C *his*+14C *leu*	
		S.F.*	P.F. (%)*	S.F.	P.F. (%)	S.F.	P.F. (%)
Koji-extract	Ballg. 1°	1:2	4.8	1:1	2.7	1:5	2.4
	10°	1:2	0.2	3:1	0.8	1:6	0
Wort	1°	1:1	0.7	1.5:1	1.1	1:2	0.8
	10°	1:1	0	1.6:1	0.2	1:1.5	0

* See Table 14.

tion of both component types of nuclei upon isolation of a single conidium or single hyphal tips of the suspected heterocaryon. Pedigree culture on C.M. by single-spore analysis showed that the heterocaryons preserved on M.M. segregated out into the two component types, but about 15–20 per cent of the conidia remained heterocaryotic. It is therefore recognized that nutritional markers give more critical evidence for formation of heterocaryons than color markers because of their ability to grow on M.M.

In our later work heterocaryons were formed from two strains having different colors of conidia as well as different nutritional requirements as the first step for the production of supposed heterozygous diploid strains. The results will be described in detail in the next section of this series[50].

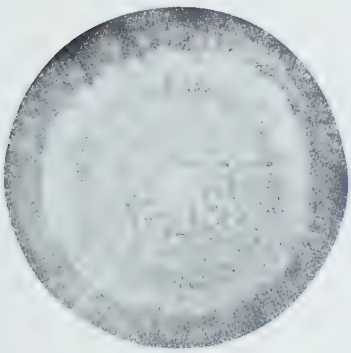
Allelism-test:

Beadle and Coonradt used heterocaryons in tests for allelism in *Neurospora*, and showed that a heterocaryon between two similar biochemical mutants was ordinarily formed when the mutant genes are not allelic, that is, are at different loci. The results were compared with the growth response of their mutants for related required substances, and manifested the route of biosynthesis of each mutant. The ability of the heterocaryon to grow on M.M. is attributed to the complementary action of the normal alleles of the two mutant genes concerned. In the course of our heterocaryon formation test using nutritional markers, such allelism-tests were carried out.

All mutants used are listed in Table 17. The tests for heterocaryon formation were the same as those described previously. Koji-extract agar (Ballg. 1°) or minimal medium, supplemented with 1/5 of the optimal concentration of the required substance were used. The ability to form a heterocaryon was first tested by transferring a colony developed after 5 days' incubation onto minimal solution. If a heterocaryon has been formed, vigorous growth takes place on the surface of the minimal solution after 2 days' incubation. Secondly suspension of 10^5 conidia/ml prepared from the mass of the colony was routinely plated onto M.M. and examined with respect to prototrophic heterocaryon formation.

Plate 5. Colonies of Component Strains and Prototrophic Heterocaryons

Component Strains



X-398 (10C *his*)

X-793 (14C *leu*)

Prototrophic Heterocaryon

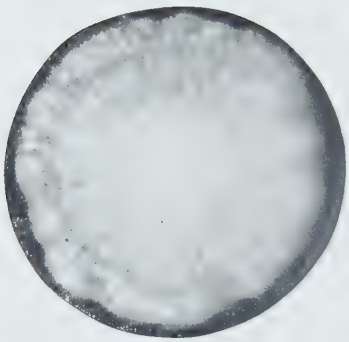
(Koji-extract)

(Minimal Medium)

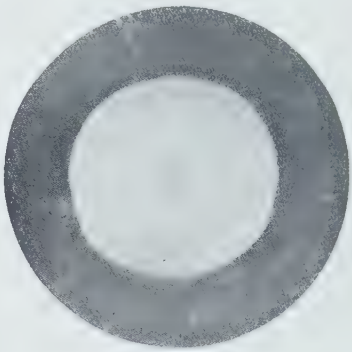
In Plates 5 and 6, the segregation of Prototrophic heterocaryon can be seen on Koji-extract plate which contain, of course, growth factors; on minimal plate no segregation is observed.

Plate 5. Colonies of Component Strains and Prototrophic Heterocaryons

Component Strains

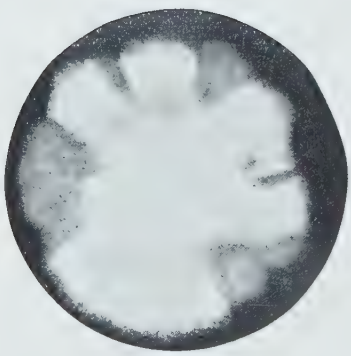


X-208 (1St *lys*)

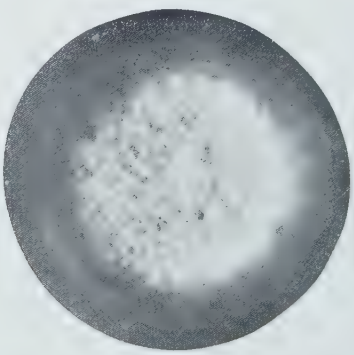


X-1125 (3MCR *his*)

Prototrophic Heterocaryon



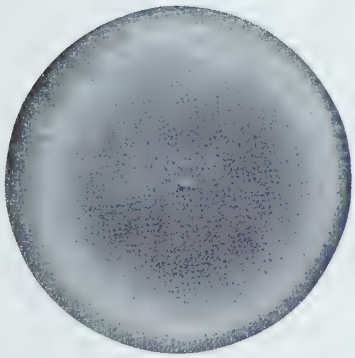
(Koji-extract)



(Minimal Medium)

Plate 6. Colonies of Component Strains and Prototrophic Heterocaryons

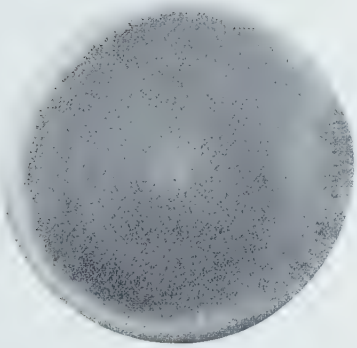
Component Strains



H123 (C *his arg*/*cit*/*or*)

L490 (C *leu* arg/cit/or)

Prototrophic Heterocaryon

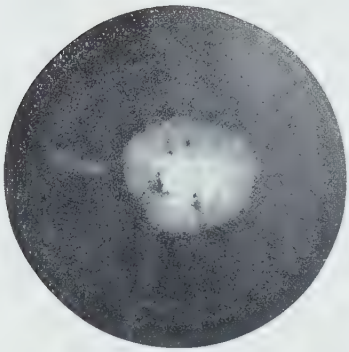


(Koji-extract)

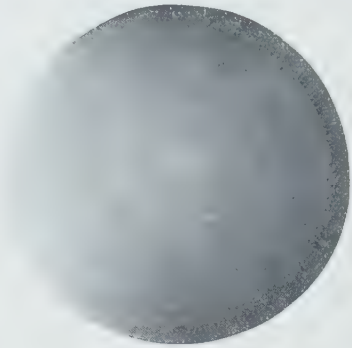
(Minimal Medium)

Plate 6. Colonies of Component Strains and Prototrophic Heterocaryons

Component Strains



H249 (CR *his meth/homo*)



L467 (St *leu/meth*)

Prototrophic Heterocaryon

(Koji-extract)

(Minimal Medium)

Table 17. Allelism-tests between Certain Biochemical Mutants of *Asp. sojae*.

Leucineless Mutants

Combinations	14C *leu*	15C *leu*	17C *leu*
X-793 14C *leu*	–		
X-573 15C *leu*	–	–	
U-191 17C *leu*	–	–	–

Histidineless Mutants

Combinations	3MCR *his*	10C *his*	11C *his*	C *leu his*
X-1125 3MCR *his*	–			
X-398 10C *his*	–	–		
X-1427 11C *his*	+	+	–	
L367 C *leu his*	–	–	+	–

Lysineless Mutants

Combinations	1St *lys*	2St *lys*	6St *lys*	20C *lys*	C *leu lys*
X-208 1St *lys*	–				
X-809 2St *lys*	+	–			
X-747 6St *lys*	–	+	–		
M-535 20C *lys*	+	+	+	–	
L520 C *leu lys*	+	+	+	+	–

Adenineless Mutants

Combinations	4C *ad/hyp*	9C *ad/hyp*	27C *ad/hyp*	b *leu ad/hyp*
X-1026 4C *ad/hyp*†	–			
X-750 9C *ad/hyp*	+	–		
M-205 27C *ad/hyp*	–	+	–	
L387 *b leu ad/hyp*	+	+	–	–

† Will grow on adenine or hypoxanthine

Methionineless Mutants

Combinations	St *leu meth*	C *leu meth/homo*	CR *his meth/homo*	29C *meth/homo*	30C *meth/homo/cys*	31C *meth/homo/cys*
L467 St *leu meth*	–					
L326 C *leu meth/homo*‡	+	–				
H249 CR *his meth/homo*	+	+?	–			
M-50 29C *meth/homo*	+	–	–	–		
M-1413 30C *meth/homo/cys*◎	+	+	+	+	–	
M-1538 31C *meth/homo/cys*	+	+	+	+	+	–

‡ Will grow on leucine and methionine or leucine and homocysteine
◎ Will grow on methionine, homocysteine or cysteine

Arginineless Mutants

Combinations	C *arg*	C *his arg*	*y arg/cit*	C *arg/cit/or*	C *leu arg/cit/or*	C *his arg/cit/or*
X-798 C *arg*	−					
H215 C *his arg*	+?	−				
Y_1-44 *y arg/cit*÷	+	+	−			
X-727 C *arg/cit/or*×	+	+	+	−		
L490 C *leu arg/cit/or*	+	+	+	−	−	
H123 C *his arg/cit/or*	+	+	+	−	+*	−

÷ Will grow on arginine or citrulline
× Will grow on arginine, citrulline, or ornithine
? The two mutants have the same growth response, but heterocaryons give rise to growth.
* Heterocaryon grows very thinly on M.M. unlike wild type (Plate 6).

By the above method, if heterocaryons form between two similar mutants, they are non-allelic; if heterocaryons do not form, the mutants are probably alleles. A negative result, the inability to form a normally growing heterocaryon on M.M., however, must be interpreted with caution. The situation does not necessarily mean that the genes are allelic. The inability to form a heterocaryon may be due to any of the following reasons: 1) Failure to obtain the suitable nuclear ratio required to attain growth. The control of nuclear ratios was shown by Dodge[5], confirmed by Beadle and Coonradt[6], and further studied by Atwood and Pittenger[51]. 2) Suppresion of wild type growth by the many mutant spores, analogous to the crowding effect found for bacteria and *Neurospora* by Grigg[52], Jinks[55], and Ryan[54]. 3) Genetic control of heterocaryosis of other genes, as reported by Garnjobst[44] for inositoless and riboflavineless strains or described by Holloway[55] for pantothenicless and lysineless strains in *Neurospora*.

Heterocaryons produced were similar to wild type, not only in their ability to grow normally on M.M., but also generally in their phenotype. However, one exception was observed in a heterocaryon between two arginineless mutants (C *leu arg/cit/or*+C *his arg/cit/or*). They are individually indistinguishable biochemically and morphologically, i.e. they have the same positive response for arginine or related substances (citrulline or ornithine) and they show the same conidial type. On M.M. the heterocaryon grows very sparsely and exhibits yeast-like colonies (Plate 6). On Koji-extract agar the heterocaryon grows normally, but the color of the colony is yellowish green, unlike the wild type. A possible explanation of this result might be pseudo-allelism with an incomplete position effect. It is reported by many workers that closely linked mutant alleles give similar phenotypes, morphologically and physiologically. Giles[42] reported that 8 inositolless mutants of *Neurospora* were produced by repeated mutation at a single locus to give a series of multiple alleles (isoalleles) but they give rise to

rare wild types in crosses. Bonner[56] also states that 5 niacineless mutants of *Neurospora* are composed of three closely linked units at the Q locus separable by crossing-over.

In *Aspergillus nidlans*, different mutants requiring biotin were tested by Roper[57] and requring adenine by Pontecorvo[58]. These pseudo-allelic mutants can not be distinguished biochemically, but recombination occurs between them at an extremely low frequency. They do not express a wild phenotype when in trans-configuration $\left(\frac{aB}{Ab}\right)$ in a heterocaryon.

The genetic relation to individual steps in arginine synthesis were analyzed by Srb and Horowitz[59] in *N. crassa*, by Bonner[46] in *P. notatum*, and by Pontecorvo[40][60] in *Asp. nidulans*. The first two species have the same blocks in biosynthesis, i.e. arginine is synthesized from ornithine through citrulline. However, citrulline is apparently not an intermediate in *Asp. nidualns*. In the course of our allelism-tests, 6 argininеless mutants of *Asp. sojae* and 3 argininеless mutants of *Asp. oryzae* were examined for interactions with arginine or related substances (Table 18). Among the 9

Table 18. Growth Response of Mutants Requiring Arginine or Related Compounds.

Mutants	Genotype	Glutamic acid	Proline	Ornithine	Citrulline	Arginine
H 123 *	C *his arg/cit/or*	−	−	+	+	+
L 490	C *leu arg/cit/or*	−	−	+	+	+
X-727	C *arg/cit/or*	−	−	+	+	+
Y_1-44	y *arg/cit*	−	−	−	+	+
H 215	C *his arg*	−	−	−	−	+
X-798	C *arg*	−	−	−	−	+
K 40 **	C *arg/cit*	−	−	−	+	+
S 12 ***	M *arg/cit/or*	−	−	+	+	+
S 17	M *org/cit/Prol*	−	+	+	+	+

* Mutants: from *Asp. sojae* K. S.
** Mutants: from *Asp. oryzae* K. B.
*** Mutants: from *Asp. oryzae* S7.

mutants, citrulline was found to be effective in 7 of them. This result does not agree with Pontecorvo's in *Asp. nidulans*.

Heterocaryons were not generally formed between mutants having the same nutritional deficiency. However, in some combinations (C *his arg*+C *arg* and C *leu meth/homo*+CR *his meth/homo*) heterocaryon formation occurred. The heterocaryons produced are phenotypically like wild type, growing normally on M.M. It might be concluded that these mutants are pseudo-

alleles or they are blocked two separate steps in the synthesis of arginine or homocysteine.

3. Segregation and Stabilization of Heterocaryons

The stability of Heterocaryotic strains, including wild strains which were isolated from nature, culture strains which were preserved in the laboratory, and heterocaryons synthesized by mixed culture of strains with different color or nutritional markers, is of interest both for the preservation of strains and as a genetic problem. We therefore studied segregation of the above strains by pedigree culture, the effect of cultural conditions on segregation, and other problems.

(1) *Segregation from Strains with Large Conidia*:

After 12 generations of pedigree culture of wild strains (*Asp. oryzae* aX, bX, and cX), the original strains produced 4 homo-types (C, M, MR and Nit) as shown in Table 2 of section I of this series[33]. Among 17 large conidial strains, 13 strains of *Asp. oryzae var. magnasporus* segregated out various colony types, including constant homo-types and inconstant hetero-types (Table 2 in section IV[34]). The above strains seem to be composed of 2–6 components and are therefore multiple heterocaryons.

An attempt to synthesize heterocaryons was made by mixing cultures of components of *Asp. oryzae var. magnasporus* SH8–4, SH31, and *Asp. oryzae* aX. Strains having a parental phenotype and multinucleate conidia were produced (Fig. 1). Unfortunately the production of multiple heterocaryons with reliable genetic markers has not yet been achieved.

(2) *Homocaryotic Multinucleate Strains*:

The 4 large conidial strains (*Asp. oryzae* A5–4, A5–7, SH15–2, and SH24–1) do not segregate out any other than their original colony types and N (no growth on Czapek's agar, but growth on Koji-extract or yeast-extract agar). Comparing the diameter of the conidia of the original strains and that of their offspring which were obtained after 4–5 generations of pedigree culture, the latter strains still have large multinucleate conidia, but a narrower size range than the parents (Table 19).

Previous investigations[34] have shown that the inconstant and the constant strains differ with respect to the diameter of conidia, the final number of nuclei in conidia, and the number of nuclei migrating into the conidia. In the inconstant strains the conidia are large (6–10μ or more), multinucleate (8–20 or more), and more than two nuclei migrate from the sterigmata into the newly formed conidia. Upon analysis of the number of nuclei migrating into the conidia, there was found no clear difference between the 13 inconstant strains and the 4 constant strains in *Asp. oryzae var. magnasporus* (Table 19); we observed 2–4 nuclei entering the conidia at the same time in both strains. Hence the above 4 large conidial strains are exceptional. As an

Table 19. Distribution of Constant and Inconstant Strains with Regards to Number of Nuclei in Conidia, Sterigmata, and Nuclear Migration into Conidia.

	Strains	Colony Types	Number of Nuclei in Conidia (%)											Sterigmata			Nuclear migration
														Double		Single	
			1	2	3	4	6	8	10	12	14	16	18	Primary	Secondary		
Constant	A5–7	MC				40	55	5									
						6	*84*	*10*								*4~6*	*1* or *2~3*
	SH15–2	Mc				3	41	38	15	3							
						2	*70*	*26*	*2*					*2~6*	*3~6*	*4~8*	*1* or *2~3*
	SH24–1	MCR					41	41	13	3	2						
							44	*35*	*15*	*3*	*3*					*4~6*	
	A5–4	MR				3	5	40	42	6	2	1	1				
						2	*5*	*33*	*22*	*19*	*12*	*4*	*3*			*4~8*	*1* or *2*
	A5–3	MCR				3	3	30	32	24	6	2					
						19	*66*	*15*								*6~8*	*2~4*
	A1–5	MCR					*24*	*60*	*14*	*2*							
Inconstant	SH13	C_5 mm		14	0	31	38	13	4								
				24	*5*	*52*	*19*									*4~8*	*2*
	H1–1	C_5 mR				22	29	33	9	4	1	1					
				38	*9*	*35*	*18*									*6~8*	*2~3*
	SH8–4	C_4 mm				3	62	24	10	1							
		C_5 mm		*3*	*0*	*50*	*47*									*4~6*	*2*
		C	*8*	*50*	*17*	*25*											
		C_2 mm	*12*	*83*	*3*	*2*											
		C_4 m					*63*	*34*	*3*							*4~6*	*2~3*
		M	*18*	*66*	*12*	*4*											
	SH31	MC					4	37	38	12	6	3					
		C_3				*2*	*14*	*55*	*20*	*7*	*2*			*2~4*	*2~6*	*2~8*	*2~3*
		C_5 mm		*3*	*1*	*14*	*15*	*20*	*7*					*2~6*	*2~6*	*4~6*	*2~4*

Roman Type Numbers: Parent
Italicized Numbers: Offspring obtained by pedigree culture

explanation of this fact the follwing reasons might be considered; 1) these strains contain heterocaryotic conidia, but do not segregate within the numbers of colonies tested because of the high nuclear-ratio; or 2) they produce

some segregants which are indistinguishable morphologically.

One of the present authors[32] has previously classified *Asp. oryzae* into three varieties according to conidial size. One of these, *Asp. oryzae var. magnasporus*, has conidia more than 8μ in diameter and includes 17 strains. In the course of the present study, 13 of these were shown to be hetero-types, and the rest were found to be homo-types in colony character. On this basis we should correct the classification of this variety, and only the 4 homo-type strains should belong to *Asp. oryzae var. magnasporus*.

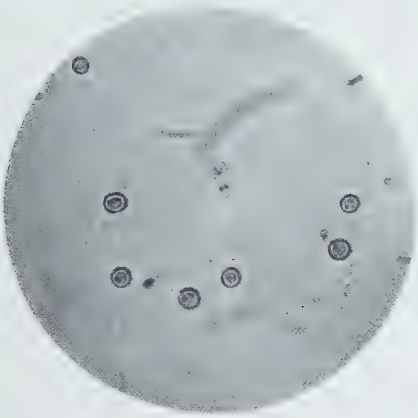
In order to resolve the above matter, the production of homocaryotic multinucleate strains was attempted. The strains treated were 2 fixed homo-types; one was a C-type, obtained from *Asp. oryzae* aX, and the other was from *Asp. oryzae* K.B. The majority of the conidia in both strains contain two nuclei. Camphor has been used most commonly as an autopolyploidy-inducing substance for microorganisms, by means of which gigas forms have been produced in yeast (Bauch[61]; Thaysen and Morris[62]; and Levan;[63-64]), in *P. notatum* (Sansome[65-66]), and *in Pasteurella pestis* (Won[67]). These authors state, without direct cytological evidence, that the gigas forms are diploid, although Sansome examined them rather precisely for conidial diameter, nuclear size, and by genetic analysis. Some work has been done in the treatment of microorganisms with colchicine (Cornman[68]; Beneke and Wilson[69]; Subramaniam and Sreepathi[70]), showing that the susceptibility of microorganisms to colchicine is very low. It is known that colchicine

Table 20. The Effects on Germination-ratio (%) and Growth-rate (Colony Diameter mm) of Concentration of Camphor.

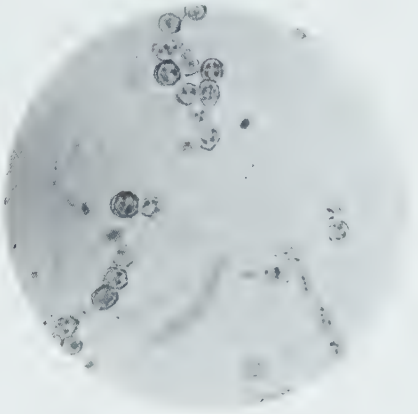
Culture		*Asp. oryzae* aX-C						*Asp. oryzae* K. B.					
		Concentrations (mg/100 ml)						Concentrations (mg/100 ml)					
	(hr)	0	30	60	150	300	450	0	30	60	150	300	450
Germination	5	38	17	0	0	0	0	72	0	0	0	0	0
	8	99	97	51	0	0	0	98	64	57	0	0	0
	24	99	97	99	98	0	0	98	97	98	97	0	0
	48	99	98	99	98	50	0	99	98	98	97	80	0
	72					98	50					98	80
	96						98						98
Growth-rate	23	9	8	5	0	0	0	8	6	4	2	0	0
	48	27	24	19	8	0	0	27	23	15	8	0	0
	72	43	38	31	18	3	0	40	39	28	16	5	2
	96	—	—	—	—	4	2	—	—	—	—	10	8
	120	66	65	60	42	5	4	65	63	49	29	18	11
	144	72	70	70	60	33	28	—	—	—	—	37	45
	240	80	80	80	80	37	35	75	75	75	75	55	55

Plate 7. Conidia Treated with Camphor and Acenaphthene

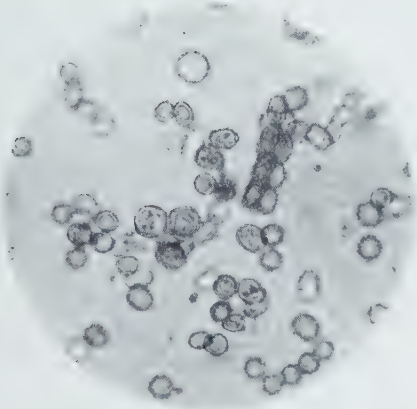
Asp. oryzae K. B.



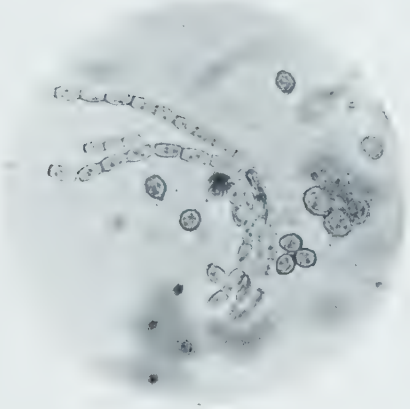
Control



Acenaphthene treatment

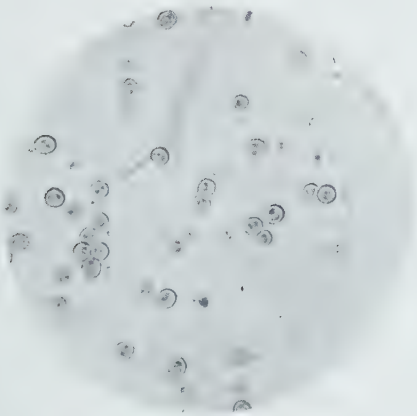


Camphor treatment

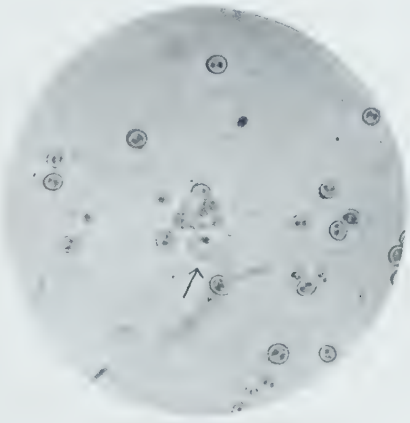


Camphor treatment

Asp. oryzae aX—S



Control



Acenaphthene treatment

generally induces the disturbance of spindle formation and of normal anaphase separation in higher plants.

Camphor treatment:

Crystalline d-camphor in different amounts (30, 60, 150, 300, and 450 mg) was dissolved in 100 ml of melted Czapek's agar, at a temperature not exceeding 45°C, and immediately poured into petri dishs. The conidia were inoculated on the camphorated media, and the plates were kept at 30°C. The germination of conidia was delayed in proportion to the amount of comphor present, i.e. germination was complete after 8 hours on the control medium, but only after 96 hours on the most highly camphorated medium. However, the ratio of germination was the same on all treated media and the control (Table 20). Colonies on media containing camphor grew more slowly than colonies on the control medium. At amounts of more than 300 mg/100 ml colonies showed a growth-rate like the extremely Restriced type even after long incubation (Table 20). A close examination of the colonies on such media also revealed changes in their height, color, and sporulation. In addition, colonies grown on media containing 300 mg and 450 mg of camphor, produced conidia in a great variety of sizes and shapes, including some with extraordinarily large diameters (8–10μ or more) and oval or tube-like form (Plate 7). On the other hand, the conidia from the control culture were uniform in size and shape. Single-spore isolations of the three lines were made from the treated samples by using a micromanipulator; 1) large globose conidia, 2) large oval conidia, and 3) rod-like conidia. The conidia of each line were successively single-spore cultured in the same way up to 3 generations and were examined microscopically with respect to conidial size and shape. Each line formed conidia of the three different shapes in similar ratios (Table 21). Concerning

Table 21. Segregation Ratios (%) Obtained by Pedigree Culture of Conidia of the Three Shapes Induced by Camphor Treatment of *Asp. oryzae* K. B.

Transfer	G*			O*			R*		
	G	O	R	G	O	R	G	O	R
T_1	76	18	6	66	34	0	60	26	14
T_2	84	15	1	63	28	9	65	25	10
T_3	71	16	3	61	33	6	68	23	9

* G: Globose O: Oval R: Rod

the size of conidia, the large formes (1 and 2 above) produced large spores in ratios of about 20 to 30 per cent in the next generation. Treatment of

conidia with vapor of d-camphor for 5 or 24 hours at 30°C gave the same result as camphorated media (containing 300 mg or 450 mg of camphor/100 ml). This shows that rapid penetration of the camphor into the cells may be an important factor in the production of giant cells. These larger conidia are all multinucleate (6–8–10 or more). However, we cannot be sure whether these conidia are polyploid because no cytological difference in nuclear size or chromosome number can be detected; this is due to the minute size of the stainable bodies.

Acenaphthene treatment:

Treatment by acenaphthene vapor was carried out in the following way. The covers of petri dishes were moistened with acenaphthene dissolved in chloroform. After evaporating the solvent, the covers were coated with crystalline acenaphthene. Conidia inoculated on the Czapek's agar plates were thus exposured to the vapor through sublimation of acenaphthene for 5–24 hours at 30°C. Acenaphthene treatment gave almost the same result as camphor treatment except for the effect on germination. A delay in germination was scarcely evident, even after 24 hours' treatment with acenaphthene. Among the large, multinucleate conidia produced by acenaphthene treatment, about 40 per cent gave large conidia in the next generation. The large conidial line obtained with camphor or acenaphthene did not segregate out any other colony type. It might therefore be concluded that homocaryotic multinucleate strains were produced by such treatment.

The conidia were treated with various solutions of colchicine (0.01 to 2 per cent), but no production of large conidia occurred.

(3) *Segregation of Green Conidia Produced by Mixed Culture of Color Mutants*:

About 250 conidia produced from a combination (Y_1+Al_3) were isolated by micromanipulator and inoculated onto a plate of wort agar (Ballg. 1°) as single spot-cultures. Green conidia segregated out into three types of colonies, i.e. the two component strains and X-type (mixed colonies, containing green, yellow, and albino sectors). The green conidia from X-type colonies were pedigree cultured as described above. The segregation ratios of green conidia were nearly constant through 3 generations (Y_1: Al_4: X=37:5:58) (Table 22). No permanently green conidia were found on plating 3×10^5 spores.

Table 22. Pedigree from Green Conidia by Single Conidium Micromanipulation.

Transfer	Segregants and Segregation Ratios (%)			No. of Cultures
	Y_1	Al_4	X	
T_1	46	8	46	237
T_2	35	3	62	237
T_3	30	3	66	235

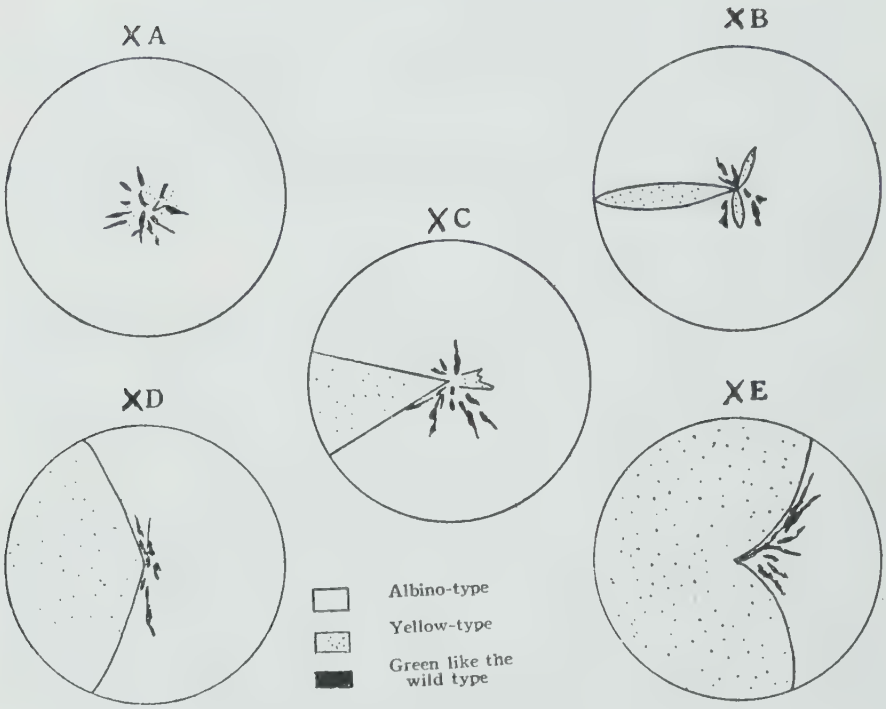


Fig. 2. Five Shapes of Sectors in X-type Colonies of *Asp. sojae*.

Table 23. Segregation from Green Conidia by Single Conidium Micromanipulation.

Combinations	Segregants and Segregation Ratios (%)			No. of Cultures
	Y_1 or Al_4	Another Component Strain	X	
Y_1+Al_1	29	5	66	50
Y_1+Al_2	36	4	60	50
Y_1+*Asp. oryzae* Albino Variant 122	81	0	19	50
Y_1+*Asp. oryzae* aX Albino Mutant Sl6A	87	0	13	50
Y_1+*Asp. candidus* B. F.	100	0	0	50
Y_1+*Asp. carbonarius* BL5–8	100	0	0	50
Y_1+*Asp. usamii* R–0635	100	0	0	50
Y_1+*Asp. ochraceus* 24–6	100	0	0	50
Al_4+Al_6	10	0	90	50
Al_4+Al_7	3	9	88	50

The X-type will be divided into 5 groups, according to the area of sectors of the albino and yellow mutants, as shown in Fig. 2. Among 416 X-type colonies examined, 94 per cent were colony types XA, XB, XC, and XD, having the larger area albino, although yellow and albino types have the same growth-rate. Each of the 5 types frequently changed into another type in the next generation, indicating that they were not fixed type.

Green colonies produced from the mixed culture of yellow (Y_1) with *Asp. candidus*, *Asp. carbonarius*, *Asp. usamii*, *Asp. ochraceus* or certain strains of *Asp. oryzae*, except *Asp. sojae* and 2 strains of *Asp. oryzae*, yielded only pure populations of original yellow mutant during the following generations. Green conidia obtained from two combinations of albino mutants (Al_4+Al_6 and Al_4+Al_7), gave rise to, however, to three types colonies (Table 23).

(4) *Stability of Prototrophic Heterocaryons*:

The prototrophic heterocaryons obtained from three combinations, (6St *lys*+14C *leu*), (6St *lys*+10C *his*), and (10C *his*+14C *leu*), were examined. These heterocaryons were single-spore cultured by the routine method on wort agar (Ballg. 1°). During 4 generations of pedigree culture, the prototrophic heterocaryons showed almost the same segregation ratios in each generation (Table 24).

Table 24. Pedigree from Prototrohic Heterocaryons (Medium: Koji-extract—Ballg. 1°)

Transfer	Combinations					
	6St *lys* + 14C *leu*		6St *lys* + 10C *his*		10C *his* + 14C *leu*	
	Segregation Ratios (%) *lys* : *leu* : X	No. of Colonies	Segregation Ratios (%) *lys* : *his* : X	No. of Colonies	Segregation Ratios (%) *his* : *leu* : X	No. of Colonies
T_1	32 : 63 : 5	852	49 : 48 : 3	1020	16 : 82 : 2	806
T_2	14 : 79 : 7	936	16 : 64 : 20	1590	36 : 60 : 4	2032
T_3	16 : 73 : 11	976	29 : 58 : 13	768	8 : 86 : 6	648
T_4	17 : 74 : 9	504	31 : 45 : 24	1336	6 : 90 : 4	1584

X—Prototrophic heterocaryon

Next, the effect of cultural conditions on the stability of heterocaryons was investigated, using the above 3 heterocaryons (Table 25). Media used were as follows: Koji-extract agar (Ballg. 1° and 10°), wort agar (Ballg. 1° and 10°), minimal medium (1/1 and 1/5 concentrations), and minimal medium supplemented with either or both of the growth factors required by the component strains. Prior to sporulation, hyphae from a colony grown on minimal medium were transferred onto each of the above 9 media. After 7 days' incubation each culture was plated onto minimal medium and Koji-extract agar. For maintaining the stability of heterocaryons, the lower

Table 25. Stability of Prototrophic Heterocaryons on Various Media.

Media	Combinations					
	6St *lys* + 14C *leu*		6St *lys* + 10C *his*		10C *his* + 14C *leu*	
	Segregation Ratios (%) *lys* : *leu* : X	No. of Colonies	Segregation Ratios (%) *lys* : *his* : X	No. of Colonies	Segregation Ratios (%) *his* : *leu* : X	No. of Colonies
Koji-extract (Ballg. 1°)	16 : 73 : 11	640	39 : 36 : 25	736	5 : 92 : 3	936
	14 : 79 : 7	936	24 : 53 : 23	600	9 : 87 : 4	648
Koji-extract (Ballg. 10°)	32 : 63 : 5	424	32 : 65 : 3	720	19 : 80 : 1	776
	35 : 64 : 1	744	42 : 43 : 15	400	5 : 94 : 1	600
Wort (Ballg. 1°)	15 : 77 : 8	616	32 : 58 : 10	944	19 : 77 : 4	488
	26 : 67 : 7	992	23 : 61 : 15	1008	27 : 63 : 10	696
Wort (Ballg. 10°)	16 : 82 : 2	576	21 : 75 : 4	720	7 : 92 : 1	720
1/5C*	32 : 48 : 20	720	31 : 41 : 28	710	37 : 38 : 25	600
	35 : 47 : 18	824	27 : 43 : 30	880	37 : 39 : 24	696
1/1C	38 : 50 : 12	600	28 : 54 : 18	472	43 : 42 : 15	404
	36 : 51 : 13	630	31 : 51 : 18	662	40 : 43 : 17	680
1/1C+Singly ** Accessory Growth Factor	+Lys		+Lys		+His	
	39 : 52 : 9	560	37 : 41 : 22	640	61 : 33 : 6	680
					73 : 21 : 6	784
	+Leu		+His		+Leu	
	13 : 82 : 5	448	25 : 60 : 15	664	1 : 98 : 1	688
1/1C+Both Accessory Growth Factors	23 : 72 : 5	352	33 : 54 : 13	632	40 : 50 : 10	600
					37 : 38 : 15	640

* C: Czapek's agar

** L—Leucine, 100; L—Lysine-HCl, 200; or L—Histidine-HCl, 100 mg/l

In most cases two experiments are reported.

concentrations of both natural and synthetic media were found to be favorable. Heterocaryons cultured on minimal medium had the highest stability. Accordingly minimal medium was chosen to preserve prototrophic heterocaryons, since it provides a selective advantage for heterocaryons. The stability of heterocaryons growing on minimal medium with added accessory growth factors was lowered. The segregation ratio of each component strain generally changed according to whether or not the growth factors required by each strain were added. The effect of adding various amounts of growth factors was not studied. However, from experiments of this series, it may be suggested that: 1) heterocaryons break down into component homocaryotic strains but are continually reconstituted through hyphal anastomosis, and 2) selection occurs between the two types of nuclei within the heterocaryotic hyphae or among the three types of hyphae, including the heterocaryotic and the two homocaryotic hyphae. Beadle and Coonradt[6]

state that the nuclear ratios vary rather widely for different combinations and also in different determinations within one combination but whether this finding was due to a methodological artifact is not certain (Prout *et al*[71]). The selective adjustment of nuclear ratio was studied by Ryan[41], by Ryan and Lederberg[72], and by Jinks[73]. They showed that the selection phenomenon was influenced by genetic background and by environment. Aside from selection, Garnjobst[44] established the existence of two independently segregating genes (het-1 and het-2) concerned in the formation and/or maintenance of a stable heterocaryon. Holloway[54] described that, in the process of heterocaryon formation between pantothenicless and lysineless mutant strains, 4 genes not only control whether or not a heterocaryon will be formed, but also modify the type of heterocaryotic growth. One of the 4 genes, gene Y, is concerned with the maintenance of the heterocaryotic condition. However, it is not known whether these are specific in their action for the particular mutants concerned.

At any rate, heterocaryons in *Asp. sojae* are unstable and even when preserved on minimal medium, only 17-19 per cent of the conidia remain heterocaryotic. Almost complete stabilization was achieved by the formation of heterozygous diploid strains. These are exceedingly stable; more than 99.5 per cent of the colonies remain green and prototrophic. This result will be described in detail in a later section of this series[50].

4. Phenotype of Heterocaryons

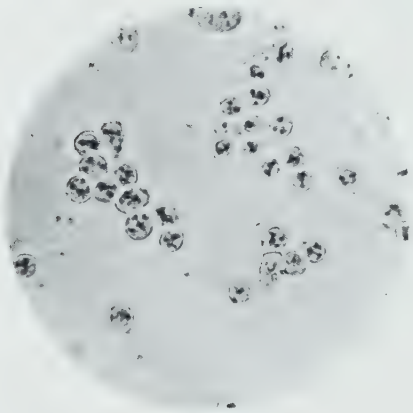
(1) *Morphological Characteristics*:

It was first described by Dodge[5] that heterocaryons between Sterile types or between Restricted types showed cultural characteristics like the normal wild type in the amount of sporulation and in general habits of growth. Heterocaryons between paired color mutants were also observed to be like wild type in color of colony. These phenomena have been attributed to complementary action due to the presence of two or more genetically different types of nuclei in a common cytoplasm.

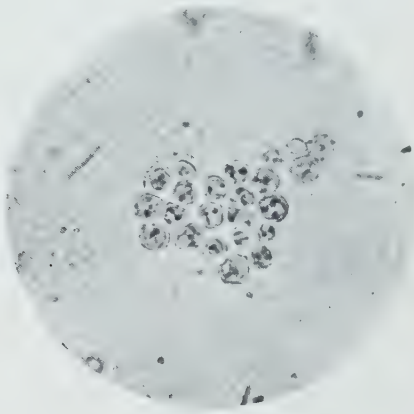
We observed previously that X-types produced by mixed culture of the two homo-types (C+M) were intermediate between the parents or like one or other parent in colony characteristics[19]. We also found that these X-types produced multinucleate conidia larger than either parent. However, whether or not the X-types are true heterocaryons was uncertain owing to a lack of good genetic markers. These interesting facts were again investigated, using prototrophic heterocaryons between single auxotrophs or heterocaryons between strains differing in color and nutritional requirements. The prototrophic heterocaryons showed a phenotype like either parent or extra heterocaryotic vigor (more abundant sporualtion, darker coloration, etc.). For a study of the size and nuclear number of conidia, heterocaryotic condia of two combinations were used: (Y_1+Al_4) and (L120 *y leu*+H158 *al his*)

Plate 8. Conidia in Homocaryotic and Heterocaryotic Cultures

Homocaryotic Conidia

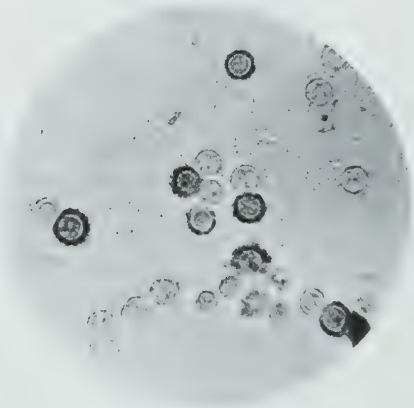


Yellow mutant (Y_1)



Albino mutant (Al_4)

Heterocaryotic Conidia



$Y_1 + Al_4$

Conidia were stained by DeLamater's formalin-mordanted basic fuchsin technique.

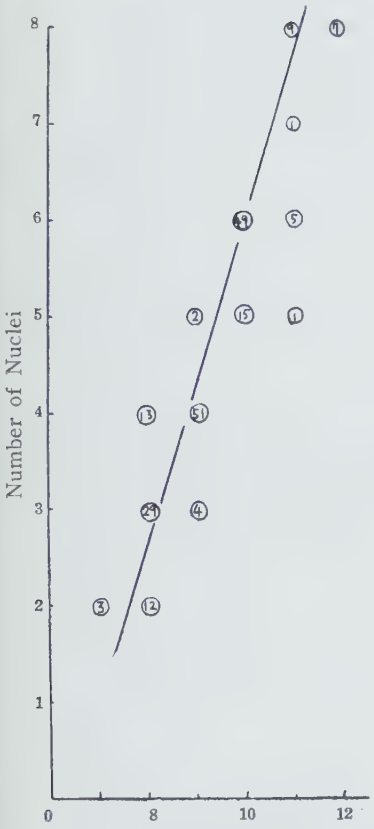


Fig. 3. The Relation of Conidial Diameters to Nuclear Number. Data include both staining and hyaline conidia from a heterocaryon (*y leu+al his*) of *Asp. sojae*.

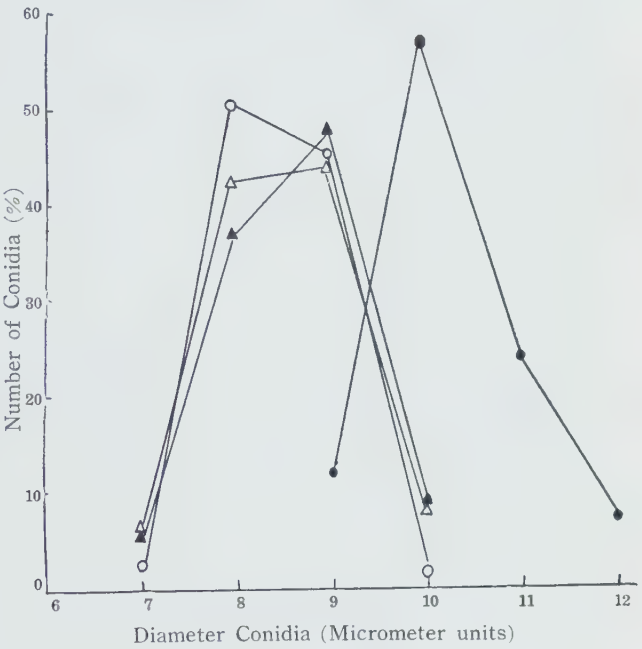


Fig. 4. Distribution of Conidial Diameters in Samples from Homocaryotic and Heterocaryotic Cultures of *Asp. sojae*.

Homocaryotic cultures:
y leu ▲—▲
al his △—△
Heterocaryotic culture (*y leu+al his*):
Hyaline conidia ○—○
Staining conidia ●—●

The conidia of the heterocaryons and the component strains were allowed to form at 30°C in petri dishes containing either minimal or supplemented medium. The measurement of conidia from 5-day-old cultures was done in dry preparations with the exception of the heterocaryotic conidia which were measured in stained preparations. There was no difference in conidial size between stained and unstained preparations. For an estimation of nuclear number, conidia, taken after 2 days' incubation, were stained by De-Lamater's[74-75] basic fuchsin technique. The conidia of heterocaryons are easily distinguishable from those of the component strains, since the conidial walls of the former stained darkly, while on the other hand, those of the latter were hyaline (Plate 8). Heterocaryotic conidia are clearly larger than

Table 26. Proportions of Conidia Containing less than 4 Nuclei.

Replicate	*Asp. sojae* K.S. Wild type (P)	Yellow Mutant (Y_1)	Albino Mutant (Al_4)	Green Conidia from Mixed Culture Y_1+Al_4 (X)	Totals
1	79	80	89	45	293
2	76	75	95	33	279
3	63	75	90	32	260
4	61	75	88	28	252
Totals	279	305	362	138	1084

Analysis of Variance

Factors	S.S.	D.F.	M.S.	F	F′
Difference between Strains	6798	3	2266	103**	26.3 *
Between P, Y_1, and Al_4	902	1	451	20.5**	5.24**
Between group (P, Y_1, Al_4) and X	5896	1	5896	268**	68.6
Replicates	258	3	86	3.90	1.00
Strains × Replication	198	9	22	1.00	
Total	7254				

Experiments were repeated 4 times. Just 100 conidia of each strain were measured at random in every replicate.

* shows significance at $P<0.5$

** shows significance at $P<0.1$

the conidia of the component strains, and are multinucleate (4–8) (Figs. 3, 4, and Table 26). The number of contained nuclei is directly proportional to conidial volume. The mechanism of increase in conidial size and nuclear number in heterocaryotic conidia has already been discussed[76].

Pontecorvo and Gemmell[9] state that the non-conidial character is dominant to the conidial one, since heterocaryons between strains with these two characters fail to produce conidia. The dominance relation between a mutant gene and its normal allele was studied by using double auxotrophic mutants with these results: 1) all heterocaryons between a Restricted type strain (H249 CR *his meth*/*homo*) which is a test strain, and 6 normal strains showed normal growth-rates; 2) all phenotypes of heterocaryons between a Sterile strain (L467 St *leu meth*) and 3 conidial strains were conidial (Plate 6); 3) heterocaryons between color mutants (L415 *y leu nic*+L387 *b* leu ad/hyp) or between color mutants and mutants with green color like wild type gave rise to normal colored conidia, approaching those of wild type; and 4) all heterocaryons between these auxotrophic mutants were prototrophic, like the wild type. From the above results it seemed justifiable to conclude that all the mutant properties concerned are recessive to the alternative wild type condition.

(2) *Physiological Characteristics*:

While genetic studies of heterocaryosis have been made by many workers, little work on physiological properties seems to have been undertaken except for that of Lindegren and Andrews[36], and Yuill[77-78]. Lindegren and Andrews attempted to improve penicillin production by using mixed cultures of *P. notatum*, but their studies failed to have immediate practical significance. This might have been because they did not use strains with proper genetic markers. Yuill reported for mixed morphological mutants of the *Asp. niger* group that citric-acid production equalled that of one or the other component strains or was intermediate. More recently the ability of heterocaryons between 12 color mutants of *Asp. fonsecaeus* to produce citric-acid was studied by Ciegler and Vejina[79] and the following results were obtained. When two relatively high-producing mutants were combined, the resulting heterocaryon produced less acid than either of the parents. When one relatively high-producing and one relatively low-producing mutants were combined, the resulting heterocaryon gave yields intermediate between those produced by the two mutant cultures. Heterocaryons resulting from two relatively low-producing strains gave yields higher than those of either component mutant. Heterocaryotic vigor of use in practical application was not found in the above studies.

Work on the improvement of Koji-molds was continued in our laboratory. The variation of enzyme activities (amylase and protease) in Koji-molds under heterocaryotic control was investigated with regard to the following three points.

1) Enzyme Activities of Heterocaryons—These were estimated in a heterocaryon between auxotrophic mutant (10C *his*+14C *leu*) of *Asp. sojae* K.S. The two mutants can not grow on minimal medium or wheat bran-medium which is used for the estimation of enzyme activities. Consequently they were cultured on wheat bran-medium supplemented with 3.8×10^{-3} mol growth factors. In protease activity, the leucineless mutant is weak (0.073), while the histidineless mutant is strong (0.640). In amylase activity both mutants are weak (7.84 and 3.3). The wild type has an amylase activity of 15.2 and a protease activity of 0.803. Amylase was estimated by the Okazaki[80] method and protease by microkeldahl method or by the amount of increase in the trichloroactic-acid-soluble nitrogen of casein[81-82]. The heterocaryon can grow on wheat bran-medium, so this medium, like minimal medium has a selective advantage. The heterocaryon showed protease activity like the highest component mutant (0.652) and amylase activity intermediate between the two mutants (4.8).

2) Enzyme Activities of Segregants from Heterocaryons—A heterocaryon was obtained from a single hyphal tip, isolated by micromanipulator, resulting from anastomosis of single conidia of *Asp. oryzae* K.B. and S7.

Table 27. Enzyme Activities and Colony Types of the Strains Used for the Production of Heterocaryons.

Strains		Amylase Activity		Protease Activity		Colony
		Symbol	Range	Symbol	Range	Types
Asp. oryzae s. str	S7	A	40.6~45.8	p	0.64~1.74	Mc
	S1-2	A	38.1~45.3	p	0.85~2.70	M
	B	A	36.8~39.0	p	0.52~1.58	M
	S4-15	A	33.1~39.9	P	2.62~3.42	MC
	H	AM	16.5~20.9	p	0.50~2.38	M
	K.B.	a	11.2~14.4	P	2.06~3.54	C
Asp. sojae	SH10-1	a	6.2~ 9.6	P	2.86~3.60	C
	SH10-2	a	4.2~ 6.8	P	1.56~2.66	C

Both enzyme activities were estimated for fifty cultures of each strain. Amylase activity: Glucosidic bonds hydrolyzed, % in 15 min. under the condition as follows: 2% soluble starch 10 ml, McIlvaine buffer (pH 4.8) 2 ml, mold bran extracts (1:10) 1 ml, 30°C, reducing power was evaluated by Fehling-Lehmann-Schoorl method. Protease activity: 1.5% casein solution (pH 6.5), mold bran extracts (1:10) 0.2 ml, 37°C 120 min., 10% TCA 5 ml added and filtered. The protease activity was represented in terms of mg of nitrogen in 5 ml of the filtrates, measured by microkeldahl method.

Symbol: Capital letter (strong in enzyme activity), Small letter (weak), and M (medium).

Table 28. Enzyme Activities of Mixed Cultures.

Group		Combinations	Amylase Activity	Protease Activity
I	A	S4-15 + S1-2	28.9 ~ 35.9	1.28 ~ 3.20
		S4-15 + SH10-3	27.3 ~ 31.5	1.10 ~ 2.20
	B	S4-15 + S7	28.9 ~ 38.0	1.52 ~ 3.00
		S4-15 + B	23.7 ~ 30.9	0.86 ~ 1.26
		S1-2 + B	33.3 ~ 34.6	0.89
		S1-2 + S7	13.9 ~ 29.4	1.87 ~ 2.35
	C	S4-15 + SH10-1	29.7 ~ 31.5	1.74 ~ 2.43
II	D	S4-15 + H	29.5 ~ 30.6	1.77 ~ 2.28
		S7 + SH10-3	29.5 ~ 31.4	1.04 ~ 2.50
		S7 + H	29.3 ~ 39.3	1.02 ~ 2.09
		S1-2 + SH10-1	14.8 ~ 23.8	2.42 ~ 2.94
		SH10-3 + SH10-1	4.7 ~ 7.3	2.65 ~ 2.90
		SH10-3 + B	30.8 ~ 34.0	1.11 ~ 2.40
		SH10-1 + B	23.0 ~ 32.3	0.85 ~ 1.18

	E	SH10-1	+ H	—	—
		S1-2	+ K.B.	21.5 ~ 28.2	1.28 ~ 1.99
	F	S1-2	+ SH10-1	14.8 ~ 23.8	2.42 ~ 2.94
		K.B.	+ B	22.8 ~ 29.0	1.98 ~ 2.16
III	G	S7	+ B	14.7 ~ 17.2	1.70 ~ 2.65
		K.B.	+ SH10-1	14.2 ~ 16.1	2.45 ~ 3.15
		K.B.	+ SH10-3	13.9 ~ 14.6	2.57 ~ 2.86
		B	+ H	16.0 ~ 17.9	1.08 ~ 1.26

Table 29. Segregants from Mixed Culture and Their Enzyme Activities.

Combinations	Enzyme Activities			Colony Types	Number of Cultures
	Symbol	Amylase	Protease		
S4-15 + S7 AP Ap	AP	40 ~ 44	3.1 ~ 3.7	MC	11
	AP	34 ~ 40	2.5 ~ 3.5	CmR	2
	AmP	25 ~ 30	3.2 ~ 3.7	M	2
	Amp	16 ~ 26	0.6 ~ 2.0	Cm	25
S7 + S1-2 Ap Ap	AP	34 ~ 41	3.0 ~ 3.4	MC	3
	APm	34 ~ 37	1.3 ~ 2.2	MC	8
	AmP	28 ~ 32	3.2 ~ 4.0	M	8
	ap	8 ~ 12	0.5	MR	1
	aP	11 ~ 15	2.1 ~ 3.6	C	22
S7 + K.B. Ap aP	Ap	37 ~ 41	1.2 ~ 1.5	MC	5
	aPm	9 ~ 12	1.5 ~ 2.2	C	10
	AmP	26 ~ 30	2.6 ~ 3.4	M	5
S4-15 + K.B. AP aP	AmPm	23 ~ 24	2.3	Cm	14
	AmP	27	3.2	CmR	12
	aP	13 ~ 16	2.5 ~ 3.0	C	20

* See Table 27.

The former strain is C-type, a weak producer of amylase (5.68), and a strong producer of protease (0.267); on the other hand, the latter strain is M-type, a strong producer of amylase (37.18), and a weak producer of protease (0.026). The heterocaryotic conidition was confirmed by the segregation of the three colony types, C, M, and X. Among these the C-and M-types had activities similar to the parents. The X-type was intermediate between the parents morphologically, but like one parent or the other physiologically.

3) Possibility of Producing Superior Strains by Heterocaryosis—Mixed cultures of various strains used for making "Tane Koji" were re-examined from the point of view of heterocaryosis. Sakaguchi, Akiyama, and

Ishitani[22] obtained 22 heterocaryons by mixed culture, in various combinations, of 8 strains of Koji-molds. Enzyme activities and colony types of the strains used are shown in Table 27. Table 28 shows the enzyme activities of mixed cultures. There seem to be three gorups: Group I, weaker than either parent in combination of strong enzyme producers; Group II, intermediate between the parents; and Group III, like one parent or the other. Strains superior to both parents have not been obtained in any combinations, and in general inferior strains have been produced. Our results are nearly the same as those of Ciegler and Vejina[79].

As the next step, about 1000 segregants from the 22 mixed cultures were examined enzymatically. However, no strain producing enzymes with high potencies was obtained. One interesting fact was that some test strains (S4–15, S1–2, and S7) used in mixed cultures, always produced

Table 30. Number of Conidial Nuclei and Variability of Induced Morphological Mutants Obtained from a Homo-type Strain (C-type) of *Asp. oryzae* aX.

Colony Types	Strains	Number of Nuclei	Segregation of Each Mutant in UV_1 and UV_8*															
			C	M	MC	St	R	Cm	Nit	Ol	A	B	Y	M	H	Ye	N
Parent	C0	1~4	89 *90*														
Almost-normal	C1	1~4	70 *80*														
Restricted	C5	1~4	 *2*				50 *50*										6 *4*
Sterile	C10B	1~4		*50*				62 *10*									5 *4*
Reduced Nitrate	C11A	1~8	4 *17*		2 *4*			2 *7*	50 *32*								
	C11D	1~8	2 *17*						50 *33*								
	C11E	1~8 or more				*1*			72 *49*								8 *10*
Heavy	C4	1~4													90 *85*		
Olive	C8	1~8		2 *2*	4 *50*					44 *5*							 *2*
Albino	C16A	0~3									70 *80*						20 *15*
	C16B	0~4	 *51*								70 *9*						
	C16C	1~8	5 *68*	1 *19*	15 *15*						50 *10*						 *3*
Floccose	C24	1~8	*4*	70 *32*	3 *4*		4 *62*										
	C27A	1~10		6 *42*	25 *78*												5 *4*
Yeast	C 9	1~4		*15*				3 *10*									7 *5*
Maroon-exudate	C 7b	1~2												90 *80*			3 *2*

* Roman Type Numbers: UV_1; Italicized Numbers: UV_8.

Table 31. Number of Conidial Nuclei and Variability of Induced Morphological Mutants Obtained from a Homo-type Strain (M-type) of *Asp. oryzae* aX.

Colony Types	Strain	Number of Nuclei	Segregation of Each Mutant in UV_1 and UV_8*											
			C	M	MC	Mc	R	St	Nit	CN	MN	B	Y	N
Parent	M0	1~ 4		92 *80*			1 *4*	1						6 *5*
Almost-normal	M1	1~ 4		70 *95*										3 *5*
	M2	1~ 4		80 *80*				*2*						*3*
Restricted	M9	1~ 4		*1*			80 *59*							4 *2*
	M12	1~ 4		*55*			60 *25*							3 *10*
Reduced Nitrate	M11	1~10			2 *6*				60 *20*		20 *62*			*10* *12*
	M13	1~ 8							50					3
	M17	1~10 or more	*70* *28*			2 *4*		*34*	50 *32*	8	15			*5*
S-Normal	M21	1~ 4	80			*96*								*4*
S-Restricted	M15	1~ 6					80 *59*							*1*
	M20	1~ 6					90 *60*							
Brown	M14	2~ 8 or more				5 *6*	3 *2*					49 *50*		3 *3*
Yellow	M18	1~ 4		*3*		*64*							50 *34*	3
X	M16	2~ 8	30 *25*	45 *30*	2 5									15 *4*

* Roman Type Numbers: UV_1; Italicized Numbers: UV_8.

segregants differing from the parents in both colony type and enzyme activities. These strains seem to be able to show the effect of heterocaryosis in their segregants (Table 29). Whether cytoplasmic factors are involved is not yet uncertain.

The acid-producing property (Kojic acid) of Koji-molds has been investigated by Ikeda[83].

5. Induction of Heterocaryotic Condition by UV Treatment

The heterocaryotic condition was usually established by hyphal anastomosis or spontaneous mutation in a homocaryotic line but the induction of heterocaryosis by UV irradiation of homocaryotic strains was also achieved. A preliminary report[23] has already been presented in the Journal of the Agricultural Chemical Society of Japan (1952).

Strains used:

C- and M-types, obtained from *Asp. oryzae* aX by 5 generations of pedigree

culture, were treated with UV. Both of these are homo-types as shown by the lack of segregation of other colony types. Representative induced morphological mutants, including 15 strains from the C-type and 13 strains from the M-type, were examined for conidial nuclei and strain variability. Tables 30 and 31 show the results. The UV treatment and mutant colony types have been fully described in section II of this series[84].

(1) *Nuclei in Conidia of Mutants*:

The conidia of mutants were stained by the Feulgen[24] technique and by DeLamater's[74-75] technique, described minutely in section IV of this series[84].

(a) The conidia of some mutants (Almost-normal, Restricted, Sterile, Heavy, Yeast, and Maroon-exudate) have been found to contain 1–4 nuclei like their original strains (C- and M-types).

(b) The conidia of other mutants (Reduced nitrate, Olive, Mycelial, Brown, and X[MC]) are multinucleate (2–8 or more).

(2) *Variability of Mutants*:

Mutants (UV_1) obtained immediately after UV treatment and mutants (UV_8) transferred on Czapek's agar 8 times at intervals of 3 months, were subjected to single-spore culture.

1) Among mutants belonging to group (a), Almost-normal (C1, M1, and M2), Heavy (C4), Albino (C16A), Maroon-exudate (C7b), and S-restricted (M15 and M20) were stable in morphological characteristics even through 8 generations of successive culture. They did not segregate any other colony type. However, Sterile (C10B), Yeast (C9), and some of Reduced nitrate (C6, M9, and M12) were unstable and gave rise to the parent types by reversion.

2) Mutants belonging to group (b), with the exception of a Reduced nitrate (C11E), were very liable to segregate out the original C-and M-types, and the X-type, in addition to each mutant.

From the above facts it is obvious that heterocaryotic strains can be produced by UV irradiation of homocaryotic strains.

A mutant strain (C11E) has multinucleate conidia, but gives no segregation of any other colony type in UV_1 and UV_8. Hence it is assumed that the mutant is a homocaryotic multinucleate strain.

SUMMARY

1. The frequency of hyphal fusions is strongly affected by various conditions; the main factors have been investigated.

a) The frequency of fusions is proportionate to the density of conidia per unit volume of the medium; the higher frequencies were obtained when

conidia were less than 10μ apart.

b) The concentration and the reaction of the media are important factors; optimal concentrations are between 1/5 and 1/20 of the original concentrations, and optimal reactions are between pH 5.4 and 6.0.

c) As concerns the effect of genetic conditions on anastomosis, there was scarcely any difference in the frequency of fusion between the test strain, *Asp. oryzae* K.B., and different strains of Koji-molds, but there were considerable differences in interspecific and intergeneric crosses. In the Aspergilli, *Asp. tamarii*, *Asp. ochraceus*, *Asp. wentii*, and various black *Aspergillus* species gave much higher frequencies of anastomosis than other species. In the Penicillia, only the Monoverticillata and some members of the Biverticillata anastomosed with the test strains in comparatively low frequencies.

2. The use of genetic markers (size of conidia, color of conidia, and nutritional requirements) has been examined for their usefulness in the detection of heterocaryons.

a) The large size of conidia produced in multinucleate strains such as Koji-molds proved useful in detecting heterocaryons.

b) The use of conidial color only as a criterion, without genetic confirmation, does not give reliable results.

c) Double nutritional markers give critical evidence for the formation of heterocaryons.

3. Allelism-tests were carried out by using various mimic mutants and the presence of pseudo-allelism was suggested for an argininеless locus. The genetic steps in arginine synthesis were in accordance with those in *N. crassa* and in *P. notatum*, but nor in *Asp. nidulans*; arginine is synthesized from ornithine through citrulline.

4. Heterocaryotic strains are unstable and segregate out into their component strains. The segregation ratios are affected both by genetic back-ground and cultural conditions.

5. The phenotype of heterocaryons between two mutants is generally like that of the wild type, differing from the component strains in both morphology and physiology.

a) The increase of conidial size and nuclear number of in heterocaryons is a remarkable characteristic of the Koji-molds.

b) Heterocaryons obtained by mixed culture in various combinations showed enzyme activities that were weaker than either parent, intermediate between them, or like one or the other.

6. Heterocaryosis was induced by the UV treatment of homo-type strains in *Asp. oryzae* aX without hyphal fusion.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the valuable advice and criticism of Professor B. Wada, Professor, F.J. Ryan and Assistant Professor Y. Ikeda. They are also indebted to Dr. N. Iguchi for supplying the mutant strains and to Mr. H. Akiyama, Mr. S. Takamori, Mr. M. Kuboyama, and Miss Y. Kusama for their contributions to some of the experiments.

REFERENCES

(1) DEBARY, A.: *Vergleichende Morphologie* und *Biologie der Pilze, Mycetozoen' und Bakterium* 558 (1884).
(2) HANSEN, H. N. and SMITH, R. E.: *Phytopathology* **22**, 953 (1932).
(3) ——— and ———: *Zentr. Bakt. Parasitek.* **92**, 272 (1935).
(4) ———: *Mycologia* **30**, 442 (1938).
(5) DODGE, B. O.: *Bull. Torrey Bot. Club* **69**, 75 (1942).
(6) BEADLE, G. W. and COONRADT, V. L.: *Genetics* **29**, 291 (1944).
(7) BAKER, G. E.: *Bull. Torry Bot. Club* **71**, 367 (1944).
(8) : *Mycologia* **37**, 582 (1945).
(9) PONTECORVO, G. and GEMMELL, A.R.: *Nature* **154**, 514 (1944).
(10) : *Cold Spr. Harb. Symp. Quant. Biol.* **11**, 193 (1946).
(11) WILHELM, S.: *Mycologia* **39**, 716 (1947).
(12) RAPER, K. B. and FENNELL, D.I.: *J. Elisha Mitchell Sci. Soc.* **69**, 1 (1953).
(13) BUXTON, E. W.: *J. Gen. Microbiol.* **10**, 71 (1954).
(14) SAKAGUCHI, K., ISHITANI, C., and TAKAMORI, S.: *J. Agr. Chem. Soc. Japan* **27**, 638 (1953).
(15) SAKAGUCHI, K. and ISHITANI, C.: *ibid.* **28**, 435 (1954).
(16) ISHITANI, C. and SAKAGUCHI, K.: *ibid.* **28**, 898, 903 (1954).
(17) ——— and ———: *ibid.* **28**, 939 (1954).
(18) ———, IKEDA, Y., and SAKAGUCHI, K.: *ibid.* **29**, 596 (1955).
(19) SAKAGUCHI, K. and ISHITANI, C.: *ibid.* **26**, 279 (1952).
(20) - - - and - - - : *ibid.* **28**, 445 (1954).
(21) ISHITNI, C.: *ibid.* **29**, 682 (1955).
(22) SAKAGUCHI, K., AKIYAMA, H., and ISHITANI, C.: *Meeting of J. Agr. Chem. Soc. Japan* (1952. April).
(23) ——— and ISHITANI, C.: *ibid.* **26**, 435 (1952).
(24) RAFALKO, J. S.: *Stain Tech.* **21**, 91 (1946).
(25) TASCHDJIAN, C. L. and MUSKATBLIT, E.: *Mycologia* **47**, 339 (1955).
(26) KATO, S.: *J. Penicillin* **1**, 584 (1948).
(27) IGUCHI, N.: *J. Agr. Chem. Soc. Japan* **23**, 357 (1950).
(28) HUMPHREYS, JONES, D. R.: *Nature* **173**, 37 (1954).
(29) SAKAGUCHI, K.: *J. Agr. Chem. Soc. Japan* **7**, 747 (1931).
(30) SERMONTI, G.: *Rend Ist Sup. Sanita* **17**, 213 (1954).
(31) HANSEN, H. N.: *Cited by Foster, J. W. in Chemical Activities of Fungi* (1949).
(32) SAKAGUCHI, K. and YAMADA, K.: *J. Agr. Chem. Soc. Japan* **20**, 65, 141 (1945).
(33) ISHITANI, C. and SAKAGUCHI, K.: *J. Gen. Appl. Microbiol.* **1**, 246 (1955).

(34) ——— and ———: *ibid.* **1**, 282 (1955).
(35) THOM, C. and RAPER, K.: *A Manual of the Aspergilli* (1945).
(36) LINDEGREN, C. C. and ANDREWS, N. H.: *Bull. Torrey Bot. Club.* **72**, 361 (1945).
(37) GOSSOP, G. H., YUILL, E., and YUILL, J. L.: *Trans. Brit. Mycol. Soc.* **24**, 337 (1940).
(38) RAPER, K. B.: *Amer. J. Bot.* **35**, 802 (1948).
(39) TAKAGI, Y.: *J. Agr. Chem. Soc. Japan.* in press.
(40) PONTECORVO, G.: *Adv. in Genetics* **5**, 142 (1953).
(41) RYAN, F. J.: *Cold Spr. Harb. Quant. Biol.* **11**, 215 (1946).
(42) GILES, N. H.: *ibid.* **16**, 283 (1951).
(43) GROSS, S. R.: *Amer. J. Bot.* **39**, 574 (1952).
(44) GARNJOBST, L.: *ibid.* **40**, 607 (1953).
(45) PONECORVO, G., ROPER, J. A., and FORBES, E.: *J. Gen. Microbiol.* **8**, 198 (1953).
(46) BONNER, D.: *Cold Spr. Harb. Quant. Biol.* **11**, 14 (1946).
(47) IGUCHI, G.: *J. Agr. Chem. Soc. Japan* **26**, 146 (1952).
(48) ——: *ibid.* **27**, 541 (1953).
(49) ISHITANI, C., IKEDA, Y., and SAKAGUCHI, K.: *J. Gen. Appl. Microbiol.* **1**, 272 (1955).
(50) ——, ———, ———: *ibid.* in press.
(51) ATWOOD, K. C. and PITTENGER, T. H.: *Amer. J. Bot.* **42**, 496 (1955).
(52) GRIGG, G. W.: *Nature* **169**, 98 (1952).
(53) JINKS, J. L.: *ibid.* **170**, 106 (1952).
(54) RYAN, F. J.: *ibid.* **171**, 400 (1953).
(55) HOLLOWAY, B. W.: *Genetics* **40**, 117 (1954).
(56) BONNER, D.: *Cold Spr. Harb. Quant. Biol.* **16**, 143 (1951).
(57) ROPER, J. A.: *Nature* **166**, 956 (1950).
(58) PONTECORVO, G.: *Symp. Soc. Exp. Biol.* **6**, 218 (1952).
(59) SRB, A. and HOROWITZ, N. H.: *J. Biol. Chem.* **154**, 129 (1944).
(60) PONTECORVO, G.: *Adv. in Enz.* **13**, 131 (1952).
(61) BAUCH, R.: *Naturwiss* **29**, 503 (1941).
(62) THAYSEN, A. C. and MORRIS, M.: *Nature* **152**, 526 (1943).
(63) LEVAN, A.: *Nature* **158**, 626 (1946).
(64) ———: *Hereditas* **33**, 31 (1946).
(65) SANSOME, E. R.: *Nature* **157**, 843 (1946).
(66) ———: *Trans. Brit. Myc. Soc.* **32**, 306 (1949).
(67) WON, W. D.: *J. Bact.* **60**, 102 (1950).
(68) CORNMAN, I.: *Bot. Gazette* **104**, 50 (1942).
(69) BENEKE, E. S. and WILSON, G. P.: *Mycologia* **42**, 519 (1950).
(70) SUBRAMANIAM, M. K. and SREEPATHI: *Proc. Indian Acad. Sci. Sect.* **35**, 1 (1952).
(71) PROUT, T., HUEBSCHMAN, C., LEVENE, H., and RYAN, F. J.: *Genetics* **38**, 158 (1953).
(72) RYAN, F. J. and LEDERBERG, J.: *Proc. Nat. Acad. Sci.* **32**, 163 (1946).
(73) JINKS, J. L.: *Heredity* **6**, 77 (1952).
(74) DELAMATER, E. D.: *Mycologia* **40**, 423, 430 (1948).
(75) ———: *J. Bact.* **60**, 321 (1950).
(76) ISHITANI, C., UCHIDA, K., and IKEDA, Y.: *Exp. Cell Research* **10**, 737 (1956).
(77) YUILL, J. L.: *Biochem. J.* **49**, 19 (1951).
(78) : *J. Gen. Microbiol.* **9**, 1 (1953).

(79) CIEGLER, A. and VEJINA, C. E.: *Bact. Proc.* 30 (1955).
(80) OKAZAKI, H.: *J. Agr. Chem. Soc. Japan* **24**, 88 (1950).
(81) YASUI, T.: *Meeting of J. Agr. Chem. Soc. Japan* (1955. January).
(82) SHIODA, H. and SAKAGUCHI, K.: *J. Agr. Chem. Soc. Japan* **23**, 426 (1949).
(83) IKEDA, Y.: *Meeting of J. Agr. Chem. Soc. Japan* (1955. April).
(84) ISHITANI, C. and SAKAGUCHI, K.: *J. Gen. Appl. Microbiol.* **1**, 258 (1955).

J. Gen. Appl. Microbiol.
Vol. 2, No. 4, 1956

HEREDITARY VARIATION AND GENETIC RECOMBINATION IN KOJI-MOLDS (*ASPERGILLUS ORYZAE* AND *ASP. SOJAE*)

VI. GENETIC RECOMBINATION IN HETEROZYGOUS DIPLOIDS

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Received for publication Feb. 1, 1956

The authors showed in the previous section of this series (Ishitani and Sakaguchi[1]) that the formation of green conidia in *Asp.sojae* occurred not only in heterocaryons between the color mutants, but also in cultures where the yellow mutant (Y_1) was mixed with other species of *Aspergillus* or *Penicillium*, or when the yellow mutant was inoculated onto agar containing culture filtrates of those strains. In addition, this conidial color change was induced by only altering cultural conditions, such as pH or composition of the medium. However, the green strains so obtained were extraordinarily unstable; they segregated into the component strains or reverted to the original yellow strain very readily.

Recently Pontecorvo and his co-workers[2-7] obtained new stable strains with recombined properties from balanced heterocaryons in three species *Asp. nidulans*, *Asp. niger*, *and P. chrysogenum*. Heterozygous diploid strains and segregants were produced through a "para-sexual" mechanism, involving mitotic segregation and recombination.

An attempt to obtain stable green strains in *Asp.oryzae* and *Asp.sojae* has been carried out using prototrophic heterocaryons between mutants with genetic markers for both color and nutritional requirements. The authors succeeded in producing such strains in much greater frequency by treating heterocaryons with ultraviolet light. Genetic and cytochemical analyses were then made to determine whether these new strains were heterozygous diploids.

The present paper gives details on the production of heterozygous diploid strains and on the properties of these strains. A preliminary report[8] of this study was presented in the Journal of the Agricultural Chemical Society of Japan 29; 682 (1955).

EXPERIMENTAL

1. *Synthesis of Balanced Heterocaryons*

(a) Strains and Media:

Strains with genetic markers for color and a nutritional requirement were isolated from UV-treated conidia in the course of the production of double auxotrophs. The three original strains, X-793 (14C *leu*), X 398 (10C *his*), and Y_1 (yellow mutant), are the same as those described in section III[9] of this series. They were obtained by Iguchi[10-11] after X-ray or UV treatment of *Asp.sojae* K.S, a strain which he had isolated from Soy sauce Koji.

The marked strains used in the present work are as follows: L 120 (yellow, leucineless) and L 236 (albino, leucineless), which were obtained from the leucineless mutant X-793; H 158 (albino, histidineless) from the histidineless mutant X-398; and Y_1-44 (yellow, arginine-or citrullineless) from the yellow mutant, Y_1.

The stability of these 4 strains was examined. There were no spontaneous or induced reversions among 3500-7000 colonies examined for color markers, or among 4.9×10^4-8.4×10^6 conidia tested for nutritional markers, as shown in Table 1.

Table 1. Genetic Stability of Color and Nutritional Markers in Mutants of *Asp.sojae* K.S.

Strain Number	Genotype	Colony Types	Substance Required	Stability, no reversions among the number of surviving conidia indicated			
				Colony Types		Requirements	
				No Treatment	5 Min. UV	No Treatment	5 Min. UV
Y_1	*y*	Yellow	none	9553	8611		
Y_1-44	*y arg*	〃	arginine	1768	4120	8.4×10^6	5.3×10^4
L120	*y leu*	〃	leucine	3721	3532	1.6×10^6	4.9×10^4
L236	*al leu*	Albino	〃	4891	5245	4.3×10^6	5.7×10^4
H158	*al his*	〃	histidine	6632	4998	3.5×10^6	6.3×10^4
Al_4	*al*	〃	none	5599	6339		

The media used in the course of this study are the same as those described in the first section of this series; Minimal medium (M.M.) = Czapek's agar and complete medium(C.M.) = Koji-extract agar.

(b) Formation of Heterocaryons:

The first step in obtaining stable green strains consists of the formation of heterocaryons between different strains. Mutant strains were paired in the following combinations: (L120 *y leu*+H158 *al his*), (Y_1−44 *y arg*+L236 *al leu*), and (Y_1−44 *y arg*+H158 *al his*).

The procedure by means of which heterocaryons were obtained has been described in detail in section V[1] of this series and thus will here be referred to only summarily. The conidia of the paired strains are grown in mixed culture on wort agar (Ballg. 1° pH 5.4–6.0) so as to permit the formation of heterocaryotic hyphae. After 5 days' incubation, conidia from the region of mixed growth were plated onto M.M. When a heterocaryon is formed it differs from the component strains not only in the color of the conidial heads but also in nutritional requirement; it is able to grow on M.M. on which the component strains can not grow. In the case of the above-mentioned combinations, the heterocaryons appear as green prototrophs on M.M., which is the ideal medium for the preservation of heterocaryons. Heterocaryons developed on M.M. were isolated before they sporulated. The balanced heterocaryons in *Asp.sojae* can be maintained by massive transfers or by single spore isolations from the sporulating surface because conidia of this species are multinucleate. Heterocaryotic spores from the heterocaryons produced by the method described previously[1] were between 4.5 and 7.5 per cent of the total. This is about the same as the frequency of heterocaryotic spores found in heterocaryons between single mutants (0.3–4.8 percent).

(c) Properties of Heterocaryons:

1) The heterocaryons grow well on M.M. and have yellowish-or brownish-green sporulating surfaces. 2) Upon single spore analysis of colonies grown on M.M., both component strains segregated out, and only 17–19 per cent of the conidia remained heterocaryons. 3) On C. M., or on M.M. supplemented with either or both of the growth factors required by the component strains, the heterocaryons give rise to colonies with sectors of both the white and yellow components. 4) All heterocaryotic heads were composed of conidial chains of three colors, yellow, white, and green, in different proportions.

2. *Isolation of Heterozygous Diploid*

(a) Without Treatment:

The heterocaryons of *Asp.sojae* mentioned above were examined without any treatment to determine whether they would produce stable green colonies. The conidia from heterocaryons preserved on M. M. agar slants were transferred onto M.M. in petri dishes (about 10^6 conidia per plate) and incubated at 30°C for 2 weeks. Among the colonies developing on M.M., on which only heterocaryons can grow, some strains (the supposed heterozygous diploids) appeared as whole green colonies or as green sectors in heterocaryotic colonies. In the combination *y leu+al his*, whole green colonies and sectors were not found among 5204 heterocaryotic colonies. In the other two combinations, however, *y arg+al leu* and *y arg+al his*, whole green colonies were present in frequencies of 3/2830 and 1/2412 respectively. No

sectored colonies were found. The frequency of green colonies among the total conidia plated was 2×10^{-7} and 3×10^{-6} respectively. Thus the frequency of green strains arising from balanced heterocaryons seems to vary according to the combinations of strains (Table 2).

Note: Heterocaryons are designated by the symbols indicating the differences between the component strains joined by a +sign, e.g. The heterocaryon between L120+158 is indicated as *y leu+al his*. To distinguish the green strains produced from the heterocaryons the symbols describing the component strains will be separated by an inclined line, e.g. the green strains derived from the *y leu+al his* heterocaryon are indicated by *y leu/al his*.

Tablb 2. Spontaneous and Induced Production of Green Strains(G) and Heterocaryons(X)

Heterocaryons	Treatment	Colony Types (%) on C.M.						Numbers of Colonies on M.M.		
		Y	Al	X	G	YG	Total Number of Colonies Examined	X	G	Total Examined
y leu+al his	None	23	60	17	0	0	319	5204	0	5204
	5 Min. UV	64	22	6	8	0	438	—	—	—
y arg+al leu	None	10	71	19	0	0	485	2827	3	2830
	5 Min. UV	34	46	0	5	15	315	—	—	
y arg+al his	None	16	67	17	0	0	410	2411	1	2412
	5 Min. UV	25	25	10	1	39	540	—	—	—

(b) With Camphor Treatment:

It is desirable to develop a technique for selecting diploid strains in high frequencies and therefore we have investigated various substances and conditions which might affect the fusion of nuclei. Camphor treatment of heterocaryons was used by Roper[2] in *Asp.nidulans* to obtain diploids, and this technique was also applied to *Asp. niger*. This treatment is based on the assumption that it might stimulate either the coalescence of pairs of nuclei next to one another within a heterocaryotic hypha, or the coalescence of pairs of spindles. Roper showed that camphor is effective in certain combinations of strains; however, fluctuations among the control cultures due to the clonal distribution of diploid nuclei are enormous. He concluded that camphor treatment was not essential for obtaining diploids in strains of either species. It is known that in higher plants camphor treatment results in the formation of multinucleate cells, which is accompanied by an enormous increase in cellular volume.

Levan[12-13] has made an extensive study of the effects of camphor and other substances on yeast cells. He showed that camphor influenced not only the cellular morphology but also the nuclear morphology; after a long treatment at a high concentration, C-mitosis, as he[14] terms it, is found.

This phenomenon includes a considerable increase in the chromosome number and irregularities in the mitotic mechanism (multipolar spindles, vagabonding chromosomes, formation of micronuclei, etc.). However, mitotic disturbances and the formation of large-celled types observed in yeast, which revert comparatively soon to the original type, may well be due to cytoplasmic effects. In any event, no permanent polyploid strains were isolated. In addition, a similar reaction has been induced by most of the substances in a group known for its narcotic properties. Sansome[15] found evidence from conidial diameter, nuclear size and genetic analysis that camphor-induced gigas forms of *P. notatum* were diploid. However, the mechanism of the action of camphor remains uncertain.

Attempts were made to obtain stable green strains by using camphor or acenaphthene, which are generally used as polyploidogenic agents for microorganisms. Conidia of the heterocaryon *y leu+al his*, inoculated on M.M., were treated with d-camphor vapor for 5 or 24 hours at 30°C. Conidia from colonies which developed after camphor treatment were plated out on M.M. and C.M. agar. Green colonies arose from the conidia treated for 5 hours in frequencies of 1/1368 in one experiment and 1/1530 in another among heterocaryotic colonies. The frequencies of green colonies among the total conidia plated were 2.0×10^{-5} and $3.8<10^{-5}$ respectively. Green colonies were not obtained from the conidia treated for 24 hours. Table 3 shows the frequencies of different colony types found upon plating conidia obtained after camphor treatment onto C.M., no green colonies were observed.

Prior to germination the treated conidia swelled conspicuously (14–22μ), as compared with untreated conidia (7–8μ). Camphor treatment delayed the germination of conidia, but did not affect the germination ratio.

The conidia of the same heterocaryon were treated with acenaphthene vapor 1 or 5 days at 30°C. This treatment did not give rise to any green colonies.

(c) With Ultraviolet Treatment:

The above experiments show that the frequency of stable green strains from the balanced heterocaryons, with and without camphor treatment, is very low. Ultraviolet irradiation of the conidia of heterocaryons was then used in order to try to increase the production of stable green strains.

Conidial suspensions prepared from the heterocaryon *y leu+al his* were irradiated under the conditions used for the production of double auxotrophs (Ishitani, Ikeda, and Sakaguchi[9]). After irradiation for periods of 0.5, 1, 3, 5, and 8 minutes, the conidial suspensions were plated onto C.M. and M.M., and incubated for 48 hours at 30°C. Colonies grown from conidia irradiated for 5 and 8 minutes showed on C.M. a change in the proportions of yellow-and albino-types, which are the component strains of the heterocaryon. There was a great decrease of albino types among the survivors; although the proportion of heterocaryotic colonies decreased only slightly after irradiation. This decrease was compensated for by the appearance of green colonies.

In addition, the size and number of albino sectors of heterocaryons decreased, and most heterocaryons showed slightly yellowish, green sporulating surfaces on C.M. or on supplemented medium. This might be attributed to the low survival of albino. In the material irradiated for 5 and 8 minutes stable green types occurred at a very high frequency as sectors or as whole colonies. The frequency of green colonies among survivors on C.M. ranged from 2 to 9 per cent. Their frequency in UV-treated heterocaryons was, therefore, more than 10^4 times that in untreated heterocaryons. The efficiency of production of green colonies by ultraviolet irradiation was examined for the other heterocaryons, *y arg+al leu* and *y arg+al his*. As shown in Table 2, 1–8 per cent of the survivors formed green colonies on C.M. and 15–39 per cent, yellow-green (YG) whose analysis will be described later. This new technique for the isolation of the green colonies has also been applied to another species, *Asp. oryzae* K.B. After the same UV treatment, the heterocaryon between yellow, methionineless K25-1 (*y meth*) and albino, arginine-or citrullineless K40-1 (*al arg*) strains of *Asp.oryzae* K.B. gave 1–5 per cent green colonies among the survivors. It was therefore clear that UV treatment of heterocaryotic conidia was exceedingly effective for the production of green strains.

Recently the effects of UV light on *Neurospora* heterocaryons have been investigated by Atwood[16-17]. He states that the ultraviolet damage in these conidia is largely nuclear, and that it may be reversed by an interaction between affected and unaffected nuclei. However, no stable diploids were searched for or observed. Hass, Wyss, and Stone[18] reported a stimulation of recombination of mutants of strain K 12 of *Escherichia coli* but it is not certain that their results must be interpreted that way. The stimulatory effect of UV light on *E.coli* K 12 reported by Hayes[19] has another explanation which he has shown applies to his data. James[20] working with *Saccaromyces* found an increased segregation of genetic markers after treatment with ultraviolet light and suggested that this was due to recombination in heterozygous diploids. However, the causes of the enhancement in the frequency of formation of stable green strains in *Asp. sojae* are still unknown. Nonetheless, it can be shown that it is not due to a differencial survival of green diploid conidia. First, no difference in survival after UV was observed, whence pure haploid or diploid yellow or green strains were irradiated. Second, there was an increase in the frequency of green diploids from $<10^{-7}$ to$>$ 10^{-2} after irradiation resulting in survival as high as 10^{-2}. Finally, from the date in Table 3 it can be calculated that there was an absolute increase in the number of green diploids after UV. For example, 49 green diploids were found among a total of 1.3×10^4 conidia plated after 5 minutes' irradiation, while there we none found among 1.3×10^3 unirradiated spores or among 5.3×10^3 spores irradiated for 3 minutes. The exact mechanism of diploid formation still remains, however, to be elucidated.

It is clear that the green strains obtained by the new method are not

Table 3. Comparison of Production of Stable Green Strains by Various Treatments of Heterocaryon (L120 *y leu*+H158 *al his*)

Treatment		Exp. I		Exp. II	
		Colony Types(%) on C.M.	Total Number of	Colony Types(%) on C.M.	Total Number of
Sources	Time	Y : Al : X : G	Colonies Examined	Y : Al : X : G	Colonies Examined
UV	0 Min.	17 : 67 : 16 : 0	1315	45 : 45 : 10 : 0	726
	0.5	13 : 78 : 9 : 0	750	42 : 42 : 16 : 0	630
	1	18 : 67 : 15 : 0	641	55 : 30 : 15 : 0	620
	3	23 : 61 : 16 : 0	543	69 : 22 : 9 : 0	627
	5	50 : 32 : 9 : 9	542	86 : 8 : 3 : 3	595
	8	69 : 17 : 5 : 9	497	90 : 3 : 5 : 2	581
Camphor	5 Hr.	26 : 58 : 16 : 0	2103	51 : 40 : 9 : 0	1891
	24	15 : 72 : 13 : 0	2592		
Acenaphthene	1 Day	16 : 69 : 15 : 0	2348		
	5	15 : 71 : 14 : 0	1757		

back mutants or contaminants of the wild type for the following reasons: 1) The component strains of the heterocaryons mentioned above are extremely stable for color and nutritional markers, even after UV treatment; 2) The green strains differ from the wild type in their segregative behavior and in other ways as will be described below.

3. *Properties of the Green Strains (Supposed Heterozygous Diploids)*

(a) Genetic Analysis:

Examinations as to whether the green strains are heterozygous diploids were made by genetic analysis; segregations for color and nutritioanl markers were tested. Segregation from the supposed heterozygous diploid strains of carrying the recessive properties of the component strains, either in homozygous diploid condition or in new combinations, indicates its heterozygosity and therefore its diploidy (Pontecorvo[21]).

(i) Segregation of Color Markers:

Detection of segregation of color markers can be made easily by visual inspection on C.M. because the segregants have the yellow or white conidial color of one of the component strains.

The green strains obtained after treatment of the heterocaryon *y leu*+ *al his* with camphor or ultraviolet were pedigree cultured up to the third transfer by the usual single spore method. Conidial suspensions of these strains were plated at a density of about 50 colonies per plate on C.M. Only 0.1–0.5 per cent of the colonies appearing on C.M. were yellow or albino. These could have resulted from the segregation of haploid parental *y* or *al*

Table 4. Segregants Selected on C.M. for Color Markers from Heterocaryons(X) and Green Strains(G) in Pedigree Culture
y leu/al his

Segregants	Exp. I								Exp. II		
	X*			G Obtained by UV			G Obtained by Camphor		G Obtained by UV		
	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_1	T_2	T_3
G	0	0	0	1280	745	584	1498	381	219	1064	551
G+(Y)**	0	0	0	6	1	26	2	1	1	5	13
G+(Al)†	0	0	0	54	36	33	16	6	6	37	23
X	53	185	77	0	0	0	32	0	16	0	0
Y	130	197	165	1	1	0	2	1	0	0	1
Al	126	775	170	1	0	0	2	1	0	0	0
Totals	309	1157	412	1342	783	643	1552	390	242	1106	588
% of G	0	0	0	95.4	95.1	90.8	96.9	97.7	90.5	96.2	93.7

* Conidia from heterocaryons(X) were kept on minimal agar slants were plated on minimal agar, and single colonies were picked at random to minimal agar slants. In this way pedigree cultures were established for three transfers. The same was true for the G strains.

** G+(Y): Green colonies producing small yellow spots or sectors.

† G+(Al): Green colonies producing small albino spots or sectors.

Table 5. Segregants Selected on C. M. for Color Markers from Yellow-green (YG) and Green (G) Strains in T_1.

Segregants	*y arg/al leu*			*y arg/al his*			
	Obtained by UV		As Spots on X Colonies G	Obtained by UV			As Sectors on X Colonies G
	YG	G**		YG-1	YG-2	G	
G	2	664	1331	8	1	540	846
G+(Y)	0	16	64	0	0	13	62
G+(Al)	0	32	52	0	0	27	52
G+(Y+Al)*	0	0	0	0	0	2	0
YG	140	0	0	219	165	0	0
X	0	0	0	497	458	0	0
Y	166	2	30	0	129	0	2
Al	0	1	5	0	3	0	4
Totals	308	715	1482	724	756	582	966
% of G	0.6	92.8	89.8	1.1	0.1	92.7	87.6

* G+(Y+Al): Green colonies producing small yellow and albino spots or sectors.

** G from YG

† G from YG-1

nuclei carried in heterocaryons where most of the nuclei were *y/al* (i.e.G); or they might have resulted from somatic recombination (by reduction or crossing-over, Pontecorvo *et al*[21-22]) in *y/al* nuclei. They were not studied further. All the remaining colonies retained, with the exception of rare sectors, the green color of the heterozygous diploid. We see that these green strains are exceedingly stable as compared with the heterocaryons which segregated almost entirely into the two component strains, yellow, and albino. There was no difference in segregation between the green strains obtained with UV and with camphor; furthermore, these green strains continued to segregate yellow or white at nearly the same low frequency in each transfer (Table 4).

After 10-14 days' further incubation of the above plates, one or two very small sectors or patches of one of the componet colors are found in 2-9 per cent of the green colonies. We call such mostly green colonies G+(Y) or G+(Al), and the completely green colonies are called G. This sectoring of the green strains is never seen in the wild type.

The green strains occurring spontaneously or derived by induction with ultraviolet light from other heterocaryons *y arg+al leu* and *y arg+al his* were as stable as those derived from the above hetercaryon *y leu+al his* (Table 5).

The yellow-green colonies (YG) produced by UV irradiation gave rise to a large proportion of yellow or yellow-green segregants and a small proportion of green segregants (0.1-1.1 per cent). These green types showed the same behavior in their segregation as those mentioned above (Table 5). Therefore the YG colonies seem to arise from heterocaryotic conidia where the majority of the nuclei were of the yellow parental type; only a few nuclei seemed to be of the *y/al* (i.e. G) composition.

Table 6. Segregation on C.M. after UV Irradiation of the Green Strain (*y leu/al his*).

Colony Types	Time of Treatment (Min.)						
	0	0.5	1	2	3	5	8
G	1654	509	476	483	324	507	287
G+(Y)	7	9	4	13	9	17	17
G+(Al)	45	32	21	41	35	75	59
G+(Y+Al)	0	0	1	6	2	8	10
Y	2	0	0	2	0	5	7
Y+(Al)*	0	0	0	0	1	0	0
Al	3	2	0	5	1	5	2
Total	1711	549	502	550	372	617	382
% of G	96.7	92.7	94.8	87.8	87.0	82.1	75.1

* Y+(Al): Yellow colonies with small albino spots or sectors.

The effect of ultraviolet light on the segregation of the green strains was examined by irradiating them for 0.5–8 minutes. No differences in survival were found among the green strains and other strains, including the component and wild type strains when irradiated singly, except that the survival of the albino strain was low (Fig. 1). After UV irradiation of the green strains there was a slight increase in segregation into yellow and albino types, and the proportion of mostly green colonies [G+(Y), G+(Al), and G+(Y+Al)] increased to 23 per cent, as aginst 3 per cent in the

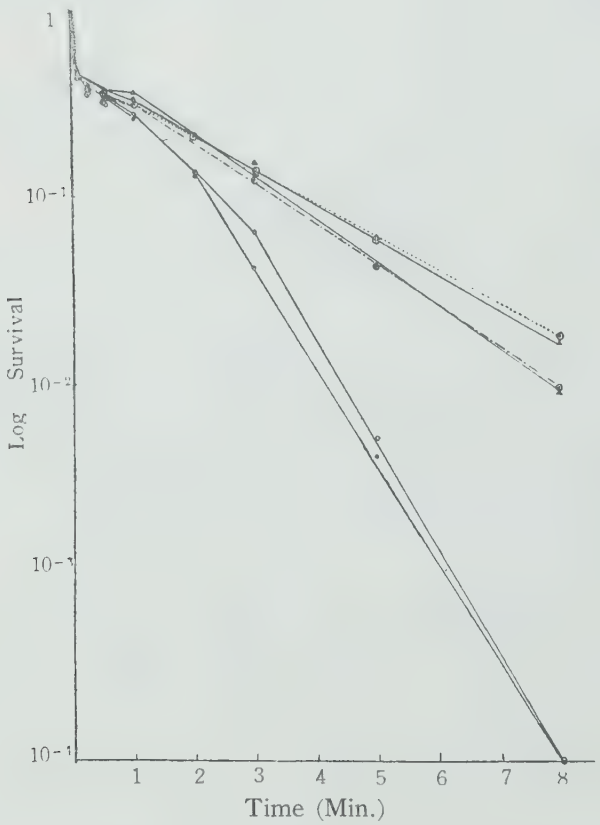


Fig. 1. Survival of the Stable Green (G) and Mutant Strains of *Asp. sojae* K. S. after Treatment with Ultraviolet Light.

◎·····◎	Wild type	K. S.
⊙——⊙	G	*y leu/al his*
△——△	L120	*y leu*
▲——▲	Y_1	*y*
○——○	H158	*al his*
●——●	Al_4	*al*

controls. As a consequence the proportion of completely green colonies decreased with UV dose. It is clear that UV stimulates the production of yellow and albino sectors in the green colonies formed but the mechanism by which this occurs is not known since the sectors and pure mutant types were not further analyzed. Table 6 shows the effect of ultraviolet light the segregation of the green strains.

All the green strains obtained originally, spontaneously or by induction, and obtained secondarily by pedigree cultures, grow on M.M. like the heterocaryons but unlike either component strain. On C.M. or a synthetic medium supplemented with either or both of the growth factors required by the component strains they formed mostly green colonies, instead of almost completely segregating out into sectors of the component types as do the heterocaryons. Spore heads were of uniformly green conidial chains, simailar to the original wild type, but the heterocaryons had mixtures of white, yellow, and green conidial chains in varying proportions.

(ii) Segregation of Nutritional Markers:

The green prototrophic strains were examined for the segregation of nutritional markers. It is more difficult to detect the segregants that differ in nutritional requirement alone, as compared with color segregants, because a visual identification could not be made. Individual colonies arising after plating on C.M. had to be analyzed for their content of auxotrophs.

Conidial suspensions of the green strains, *y leu/al his* and *y arg/al leu*, were spread on C.M. so as to give from 30 to 60 colonies per plate. Prior to sporulation, the colonies appearing after about 40 hours' incubation were spot-transferred onto plates of M.M., 20 isolates per plate. By this test a preliminary determination was made as to whether they were prototrophs or auxotrophs. The colonies that did not grow on M.M., or showed weak growth, were further tested for nutritional requirements. As shown in Table 7, all the green colonies tested were prototrophs, except for one which was leucineless, and almost all the yellow or albino segregants were prototrophs or had the same requirement as the component strains. However, two segregants from *y arg+al leu* were recombinants because they had the yellow color of *y arg* and the leucine requirement of *al leu*. There were neither double auxotrophic mutants (*leu his*, or *arg leu*) in any color combination, nor new requirements in any of the 910 isolated tested from *y leu/al his* and the 1040 isolates tested from *y arg/al leu*.

Some method of selection is required for the quantitative isolation of nutritional segregants because prototrophs may compose the greater part of the segregant clones. Prototrophs might be excluded by filtration of conidia pregerminated on M.M.[23-27], or by killing prototrophic conidia by means of the SO_2 technique[28]. As yet no such selection experiment has been done with our stocks and we may only conclude that the frequencies of auxotrophic segregants obtained are probably underestimates.

Table 7. Segregants Selected for Nutritional Markers from the Green Strains.

Segregants	*y leu/al his*				*y leu/al his*			
	Prototrophs	Requiring leucine	Requiring histidine	Totals	Prototrophs	Requiring arginine	Requiring leucine	Totals
G	755	0	0	755	781	0	1	782
G+(Y)	13	0	0	13	26	0	0	26
G+(Al)	105	0	0	105	127	0	0	127
G+(Y+Al)	4	0	0	4	0	0	0	0
Y	13	2	0	15	82	2	2	86
Al	2	0	16	18	18	0	1	19
Totals	892	2	16	910	1034	2	4	1040

If a segregant for one the markers still segregates for one or more of the remaining markers, it may be a heterozygous diploid. However, if a segregant does not segregate further, it may be either a haploid or a homozygous diploid.

We tried to determine whether or not the yellow and albino segregants, which were derived from the green strains in a low frequency (0.1–0.5 per cent), segregate further by plating their conidia on C.M. Yellow segregant strains (prototrophs) showed further color segregation; they produced albino segregants as whole colonies or sectors in a frequency of 0.1–0.3 per cent. On the other hand, no further color segregants were detected in the albino segregant strains (Table 8). No segregation of nutritional marker was observed among 504 colonies of the yellow segregant strain tested.

Table 8. Segregation of Color Markers from Yellow and Albino Segregants.

Segregants	Yellow Prototrophic Segregants from				Albino Prototrophic Segregants from			
	*y leu/al his**		*y ary/al leu**		*y leu/al his***		*y arg/al leu***	
	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Y	1804	622	579	764	0	0	0	0
Y+(Al)	67	10	57	60	0	0	0	0
Al	3	2	1	1	1014	925	623	1050
Totals	1874	634	637	824	1014	925	623	1050
% of Y	92.0	98.2	90.7	92.7				

* Yellow and albino segregants secured after UV in Exp. I in Table 4.
** Yellow and albino segregants secured from G occurring as spots on X colonies, Table 5.

(iii) Theoretical

An attempt to explain the results obtained above is here given in terms of the combination Y_1-44, *y arg* and L236, *al leu*. These component strains

differ in the following properties from one another and from the wild type.

Color: Y_1–44, yellow; L236, albino; the wild type has green conidial heads. Nutritional requirements: Y_1–44, arginine-or citrulline-less; L236, leucineless.

The two strains therefore have 4 pairs of alternative properties: albino/green; yellow/green, arginine or citrulline requirement/arginine or citrulline non-requirement; leucine requirement/leucine non-requirement. All of these differences are under genetic control because they are inherited. The heterocaryons between *y arg* and *al leu* show properties similar to those of the wild type in being prototrophic and forming green conidia but some single conidia from the heterocaryotic heads give rise to the original *y arg* or *al leu* components. It is accordingly conceivable that the properties observed are due to mutation at different chromosomal loci and that the mutant alleles are recessive to those of the wild type because the phenotypes of the heterocaryon and the heterozygous diploid are the same as the wild type. These loci may be symbolized by pairs of alleles, as follows: *AL/al*, albino; *Y/y*, yellow; *ARG/arg*, arginine requiring; *LEU/leu*, leucine requring. The alleles shown in small letters determine, when by themselves or in homozygous condition, the mutant character.

The heterozygous diploid strain may thus be described as *y/Y arg/ARG Al/al LEU/leu* (the various allelic sets are kept separate because it is not yet known whether any are linked). Segregation of the heterozygous diploids for color markers could give following genotypes:

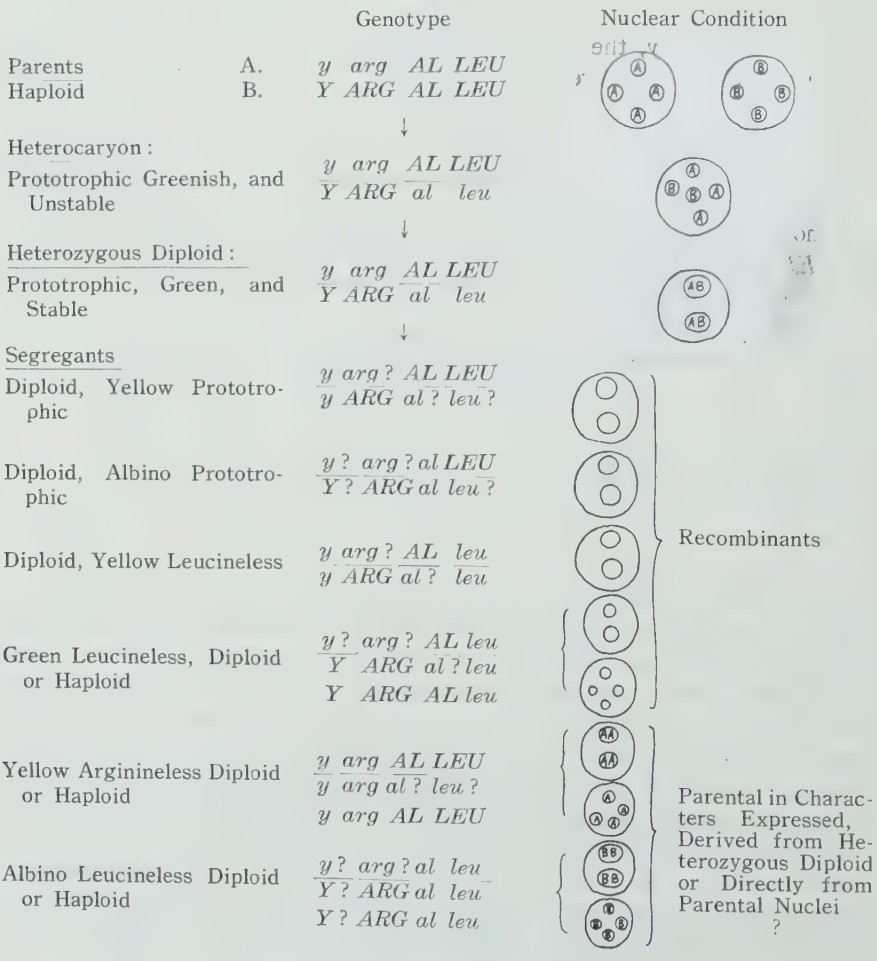
	Green	Yellow	Albino
Diploid	*y/Y AL/al*	*y/y AL/al*	*y/Y al/al*
	y/Y AL/AL	*y/y AL/AL*	*Y/Y al/al*
	Y/Y AL/al		*y/y al/al*
	Y/Y AL/AL		
Haploid	*Y AL*	*y AL*	*Y al*
			y al

Pontecorvo[4] observed that *al* is epistatic to *y* in *Asp. nidulans*. It is therefore presumed that *y/al* or *y/y al/al* would give albino conidia in *Asp. sojae* as well. Fig. 2 summarizes the results obtained in terms of these considerations.

At least three of the yellow prototrophic segregants described in Tables 5 and 7 were shown by tests (to be described in a moment) to be diploid. They could have arisen by a crossover between the *y* locus and its centromere. (Whether these diploids were homozygous or heterozygous for the other alleles was not determined, hence the recessive alleles are indicated with question marks in Fig 2; at least one dominant allele must have been present.) At least two albino prototrophs were also diploid and must have been homozygous at the *al* locus, presumably because of a crossover between that locus and the centromere. The diploid yellow leucineless segregant is homozygous for *y* and *leu*. If *y*

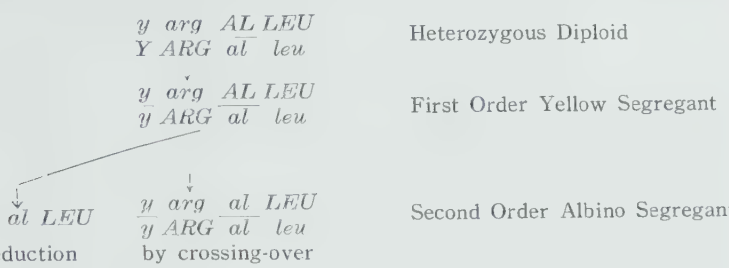
and *leu* are on the same arm of a single chromosome one crossing-over between the centromere and the proximal locus would make both homozygous; otherwise a crossing-over between each locus and its centromere would be required. The green leucineless segregant was not examined for its state of ploidy, but, whether diploid or haploid, it contains a recombination of genes achieved either by crossing-over or somatic reduction (Pontecorvo[21]). The yellow argininеless and albino leucineless segregants had the character of the parental strains. If they were haploid they might have represented parental haploid nuclei that persisted as a rare component in a heterocaryon where the major component cansisted of heterozygous diploid nuclei; or they might have been formed by somatic reduction of the heterozygous diploid nuclei. If these segregants are diploid they would have to have been formed

Fig. 2.


Genotype
Nuclear Condition
Parents A. y arg AL LEU
Haploid B. Y ARG AL LEU
Heterocaryon :
Prototrophic Greenish, and Unstable
y arg AL LEU / Y ARG al leu
Heterozygous Diploid :
Prototrophic, Green, and Stable
y arg AL LEU / Y ARG al leu
Segregants
Diploid, Yellow Prototrophic
y arg ? AL LEU / y ARG al ? leu ?
Diploid, Albino Prototrophic
y ? arg ? al LEU / Y ? ARG al leu ?
Diploid, Yellow Leucineless
y arg ? AL leu / y ARG al ? leu
Green Leucineless, Diploid or Haploid
y ? arg ? AL leu / Y ARG al ? leu / Y ARG AL leu
Yellow Argininеless Diploid or Haploid
y arg AL LEU / y arg al ? leu ? / y arg AL LEU
Albino Leucineless Diploid or Haploid
y ? arg ? al leu / Y ? ARG al leu / Y ? ARG al leu
Recombinants
Parental in Characters Expressed, Derived from Heterozygous Diploid or Directly from Parental Nuclei ?

by crossing-over.

The second order albino segregants could be either diploid or haploid, formed as follows:


y arg AL LEU / Y ARG al leu
Heterozygous Diploid
y arg AL LEU / y ARG al leu
First Order Yellow Segregant
y ARG al LEU
by somatic reduction
y arg al LEU / y ARG al leu
by crossing-over
Second Order Albino Segregant

First order albino segregants would not be expected to segregate for yellow because they were *y/Y* and became *y/y* it would not be observed; *al* being epistatic to strain would be albino in either event.

In this analysis we have followed the interpretation of Pontecorvo and his group who have carried their analysis of somatic recombination of heterozygous diploids, especially of *Asp.nidulans*, to an advanced level. (e. g. see Roper and Pritchard[20]). It is still possible that strains designated as diploid by the tests to be described are really aneuploid and perhaps the true genotype of, for example, the yellow prototrophic strain is: *y/Y arg/ARG Al/al LEU/leu*. This would be achieved by the loss of the *y* chromosome from the heterozygous diploid and not by crossing-over. A further analysis of the diploid strains and their segregants and of the hypothesis of diploidy and somatic recombination, whether it be by reduction and/or crossing-over, requires not only more genetic work but other tests as well.

(b) Cytological Studies:

Ploidy, the number of sets of chromosomes, may be inferred in many plant and animal cells from an analysis of the life cycle and confirmed by a direct count of the chromosome number. In Koji-molds, a direct chromosome count is not yet feasible. It is a well-known cytological phenomenon is higher organisms that variations in cell dimensions and in nuclear sizes are correlated with degree of ploidy. However, similar information concerning the fungi is very scare. The diploid state in imperfect fungi has been deduced generally from analysis of genetic markers present in the original haploid conidia, and from their large average conidial and nuclear size.

Pontecorvo and his co-workers used, aside from genetic analysis, only the diameter of the conidia as a diagnosis for diploidy or haploidy in *Asp. niger* and *P.chrysogenum*. Diploids have conidia about 1.3 times the diameter of the original haploids in *Asp.nidulans*[4] and in *Asp.niger*[5]; The volumes of the diploid and haploid conidia are in the ratio of 2:1. In *P.chrysogenum*[6] diploids were shown to have larger conidia, although the size of the conidia was not accurately measurable because of considerable variation in shape.

Table 9. Mean Diameter, Volume and Average Number of Nuclei per Conidium from Different Haploid, Diploid, and Segregant Strains of *Asp.sojae*.

	Strains		Diameter of Conidia (μ)	Volume of Conidia (μ^3)	Number of Nuclei per Conidium
Original	K.S. (Prototroph)		6.30	130.83	4.50
Components	L120 (*y leu*)		6.42	138.49	3.94
	Y_1–44 (*y arg*)		6.86	168.89	4.11
	L236 (*al leu*)		6.29	129.53	3.93
	H158 (*al his*)		5.99	113.04	3.85
Heterocaryons	*y leu+al his*		7.21	196.98	5.50
	y arg+al leu		7.30	203.55	5.55
	y arg+al his		7.21	196.98	5.48
Heterozygous Diploids	*y leu/al his* (Prototroph)		6.27	129.62	2.15
	〃 (〃)		6.30	130.83	2.10
	y arg/al leu (〃)		6.40	137.20	2.04
	y arg/al his (〃)		6.37	136.68	1.84
Fisrt Order Segregants	Yellow	from *y leu/alhis* (〃)*	6.21	125.93	2.27
	Yellow	〃 *y arg/al leu*(〃)*	6.40	145.22	2.08
	Yellow	〃 〃 (*leu*)*	6.32	132.11	2.20
	Yellow	〃 *y arg/al his*(Prototroph)**	6.37	135.91	2.23
	Albino	〃 *y leu/al his* (〃)*	6.25	128.38	2.18
	Albino	〃 *y arg/al leu* (〃)*	6.22	125.93	2.01

Factorial Analysis of Mean Diameter of Components and Heterozygous Diploids:

		V	D.F.	M.S.	F
between	Groups	48	1	48	$\ll 1$
within	Groups	4025	6	681	
Totals		4073	7		

Factorial Analysis of Mean Diameter of Components and Heterocaryons:

		V	D.F.	M.S.	F
between	Groups	12,385.7	1	12,385.7	15.5*
within	Groups	3,972.0	5	794.4	1.00
Totals		16,357.7	6		

* selected for nutritional requirement, see Table 7.
** selected for color, see Table 5.

Sansome[15] concluded that the "gigas" strains isolated from *P.notatum* are probably homozygous diploids partly on the following evidence: 1) The difference in conidial diameter is of the order to be expected if the original type is haploid and the gigas type is diploid. 2) The nuclei of the gigas type are larger than those of the original type. Mundkur[30] studied the interphase nuclei and cell sizes in a polyploid series of *Saccharmyces*. He

showed increments in mean nuclear diameters and ranges of variation representing each degree of ploidy in preparations of pure cultures.

The green strains of *Asp.sojae* and their segregants were further examined with regard to conidial size and the number of nuclei in the conidia.

(i) Conidial Size:

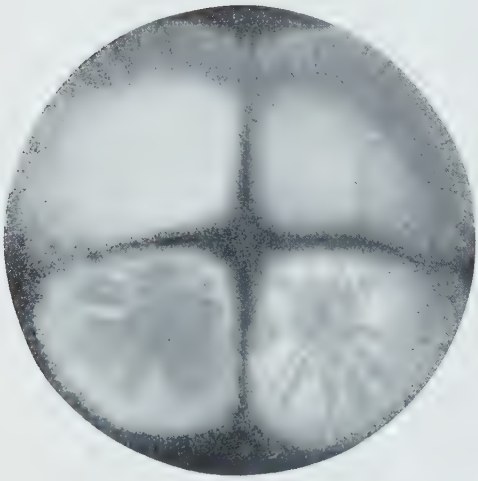
The wild type, the heterocaryons, the green strains, and their segregants were maintained on M. M. agar slants. Slants for the component strains were supplemented with the required growth factors. The conidia to be measured were allowed to form at 30°C in petri dishes containing either minimal, supplemented or complete medium. Conidia from 5-day-old cultures were measured in dry preparations. For each strain, 250 conidia were measured per preparation and this was repeated three times so that the mean diameter was based on a total of 750 conidia. The exception was the heterocaryotic conidia which were measured in stained preparations. In the previous paper of this series (Ishitani and Sakaguchi[1]) it was shown that fixation and staining did not change conidial size. There was scarcely any difference among the green strains, their segregants, the wild type and the component strains, although the heterocaryons had larger conidia (Table 9). Therefore the conidial volume of the supposed diploid strains and of the original haploid strains is found to be approximately equal. This result is not in accord with that of Pontecorvo. Nor was any difference in conidial size recognized between cultures grown on C.M., or on M.M. supplemented with the growth factors required by the component strains contrary to the findings of Huebschman[31] in *Neurospora crassa*.

(ii) Number of Nuclei in Conidia:

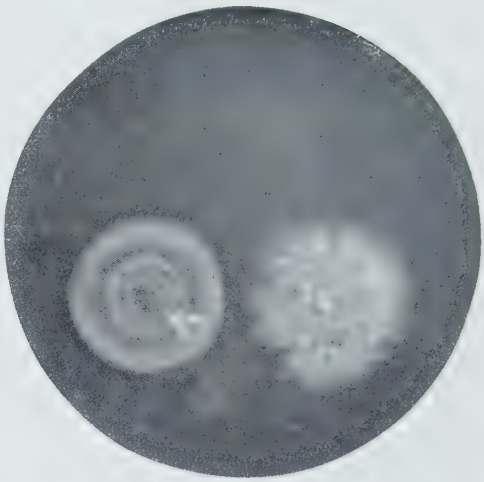
For a study of nuclear number the method previously descibed was used: Conidia, taken after 5 days' incubation on C.M. or M.M., were smeared on slides with albumen fixative, and were stained by the Feulgen technique. Samples to be examined were controlled in order to avoid any such environmental effects as culture age, culture medium, staining fluid and time, etc. In establishing the average nuclear number, three preparations were made for each strain, and the contents of 100 conidia per preparation were counted at random.

Huebshman reported that the average number of nuclei per conidium in *Neurospora* was dependent on the composition of the medium and that it increased from 2.6 on M.M. to 6.2 on C.M. He also suggested that the average number of nuclei found on a given medium was independent of the strain used and of the age of the culture. However, according to our results, the average nuclear number in conidia on M.M. was almost indistinguishable from the average value on C.M. The average number of nuclei per conidium of the wild type and component strains was 3.05–4.5; on the other hand, that of the green strains and their yellow or albino segregants was 2.01–2.27: The first group showed nearly twice as many nuclei as the second group (Table 9, Plate 2).

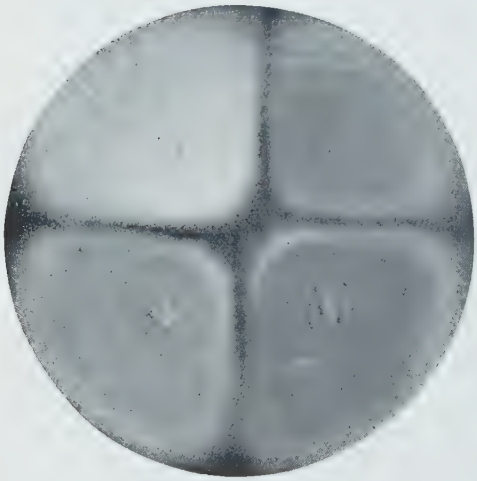
Plate 1. Colonies of Heterozygous Diploid and Segregants.



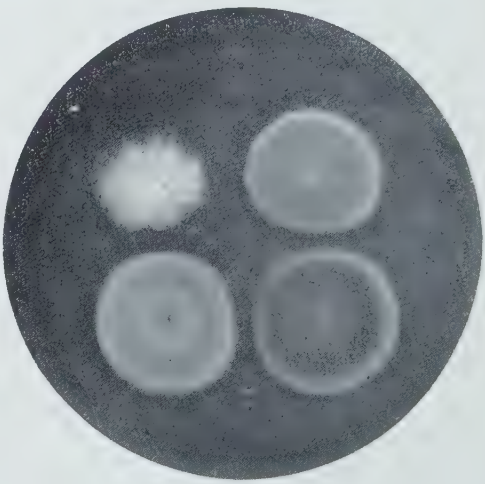
1.



2.



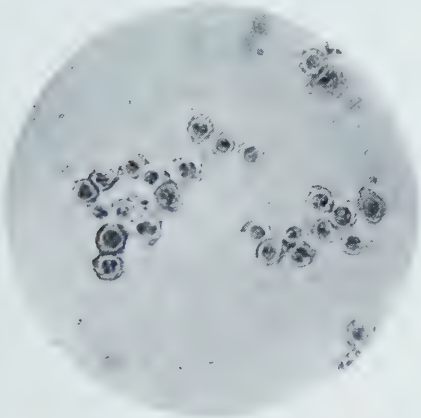
3.



4.

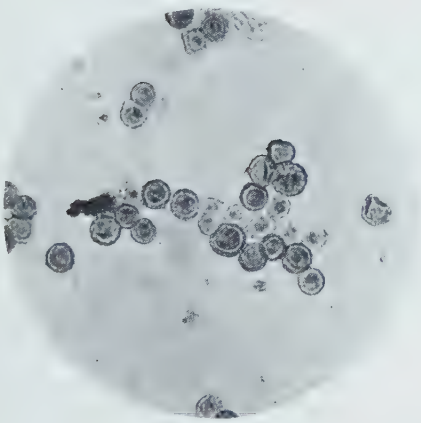
1. On Koji-extract agar: upper right L120 (yellow, leucineless), upper left H158 (albino, histidineless), lower right (heterocaryon), lower left (heterozygous diploid).
2. On minimal medium: the same strains as 1. Neither component strains grew on minimal medium.
3. On Koji-extract agar: segregants (albino, yellow, and green prototrophs), upper right (wild type K. S.)
4. On minimal medium: the same strains as 3.

Plate 2. Number of Nuclei in Conidia.

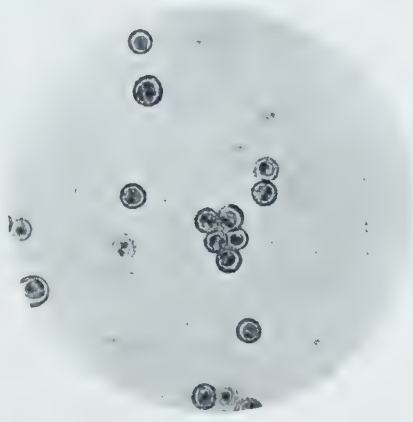


L120 (*y leu*)

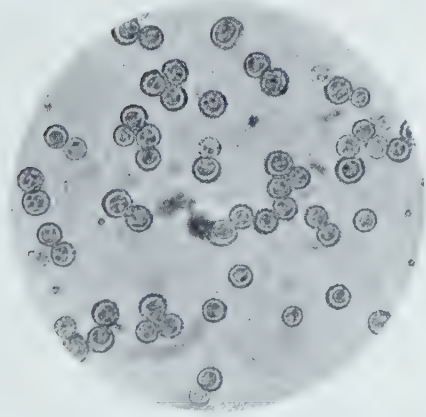
H158 (*al his*)



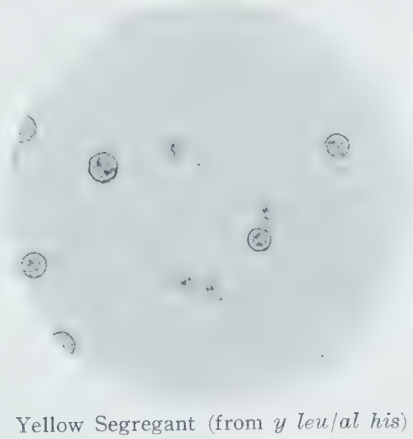
Heterocaryon (*y leu*+*al his*)



Heterozygous Diploid (*y leu*/*al his*)



Wild Type (*Asp. sojae* K. S.)



Yellow Segregant (from *y leu*/*al his*)

Upon microscopic observation the nuclei of the green strains and their segregants appeared as much larger spheres than those of the wild and component strains. This is in accordance with Sansome's hypothesis that the nuclei of the gigas type are diploid. Nuclei which are thready or chromosomal in appearance were often seen clearly in these diploid strains but no attempt was made to measure nuclear size exactly.

(c) Estimation of Desoxyribonucleic Acid Content per Nucleus:

The criteria of conidial size and nuclear size are by no means infallible in determining ploidy for the following reasons: 1) Large conidia are also produced by heterocaryons. 2) It is difficult measure conidial size because of variations in the shape of conidia and in growth conditions. 3) The true dimensions of the nuclei have not been accurately estimated since the nuclei are minute, and furthermore, chemical fixatives, such as those used, yield appreciably shrunken preparations. Accordingly, additional criteria for the reliable estimation of ploidy in fungi are required. It was observed by the Vendreleys[32], by Boivin *et al*[33], and by Mirsky and Ris[34], that the amount of desoxyribonucleic acid (DNA) per nucleus in somatic tissues was approximately constant within certain animal species, independent of the kind of tissues, and that these nuclei showed twice the level of DNA in the haploid sperm. Heagy and Roper[35] showed that conidia from haploid and diploid strains of *Asp. nidulans* had a DNA content in the ratio 1:2. Ogur *et al*[36] also stated that the DNA content per cell in yeast is consistent with an integral ratio in the polyploid series, and this character has proved the most reliable of those studied for estimating ploidy.

This subsection concerns the estimation of the DNA content of our diploid strains, of the original wild type and of the component strains. The method adopted is the diphenylamine reaction, which is a color reaction based upon the sugar components of nucleic acids. This reaction was first described by Dische[37], and was recommended for quantitative determination of DNA in animal or plant tissues with a photoelectric colorimeter by many workers (Seibert[38]; Schneider[39]; Steele *et al*[40]; etc.). Diphenylamine reacts specificially with the carbohydrate moiety of the purine nucleotides of DNA, and there exists a proportional relationship between the optical density of the blue colored product and the amount of DNA. A more extensive discussion of the specificity of the reaction and of interference from other substances will be found in the book by Chargaff and Davidson[41]. This method was employed by Heagy and Roper[35] and by Ogur *et al*[36]. A recent report by Webb and Levy[42] describes the use of p-nitrophenylhydrazine for DNA assay. It is claimed that this gives greater sensitivity, but as the dyphenylamine reaction gave good results, the other was not tried in these experiments.

Reagents: 1) Diphenylamine Reagent–1 g of diphenylamine is dissolved in 100 ml of glacial acteic acid of the highest purity, and 2.75 ml of con-

centrated sulfuric acid (nitrogen-free) is added to 100 ml of the diphenylamine solution. 2) Trichloroacetic acid (TCA) 3) Desoxyribonucleic acid (DNA)-Nutritional Biochemicals Corporation, U.S.A. P-10.2 per cent.

Materials analyzed for DNA: 1) Green conidia—the original wild type, *Asp. sojae* K.S. and supposed diploid strain, *y leu/al his*; 2) yellow conidia—the component strain, L120 (*y leu*) and the yellow prototrophic segregant, selected for color (Table 8) from *y leu/al his*; 3) albino conidia—the component strain, H158 (*al* his) and the albino prototrophic segregant, selected for color (Table 8) from *y leu/al his*.

Cultures were grown on M.M. or on C.M. at 30°C for 6 days. The conidia were harvested in distilled water containing 0.01 per cent sodium lauryl sulfonate by using a fine brush. The conidial suspensions were shaken vigorously in a vibrator for 15 minutes and filtered through a No. 2 glass filter to remove conidial chains and clumps. The conidial suspensions of each sample obtained after such treatments had on the average 90–92 per cent single spores, and concentrations of approximately $2.0–4.0\times10^7$ conidia per ml. The number of conidia in the suspensions was determined by hemocytometer counts repeated 20 times for accuracy. The average deviation of replicate counts did not exceed 5 per cent. Ten ml from about 100 ml of the suspensions were transferred to a small test tube (100 by 14 mm), centrifuged for 15 minutes at 3000 r.p.m., and the aqueous layer was discarded. By successive centrifugation of each 10 ml suspension, the conidial suspensions were brought to a cell concentration of approximately $1.0–2.0\times10^9$ conidia per ml.

The preparation of material for analysis was carried out by the method of Schneider[39]. The conidia obtained in the above manner were washed with 6 ml of 10 per cent cold TCA three times to remove acid soluble phosphorous compounds, then extracted with 6 ml of acetone to remove traces of TCA. These treatments were done at a temperature of 0°C to minimize loss of DNA from the conidia after treatment with TCA. The absorption curves of the three extractions made with TCA for each sample were determined with the Beckman spectrophotometer. They showed some nucleic acids, but the amounts were negligible. The conidia were further extracted successively by ethanol, hot ethanol (60°C): chloroform 3:1, hot ethanol: ether 3:1 (twice), and ether at room temperature, to remove phospholipids: After dehydration with ether, the samples were dried in a desiccator, which was evacuated for 10 minutes with a vacuum pump, then left for 12 hours at room temperature. The dry powder prepared in this manner was also used for estimation of the weight of conidia.

Analytical procedure:

1) Hydrolysis—Schneider[39] examined the effects of the length of heating and of the concentration of TCA on the intensity of the diphenylamine reaction. He determined that hydrolysis at 90°C for 15 minutes with 5 per cent TCA was optimal for splitting nucleoproteins. Optimal conditions for

hydrolysis of our samples were obtained with a heating period of 15 minutes or longer. No further increase in color was observed when hydrolysis was continued for 30 minutes. In addition, no color reaction occurred in the extract after a second hydrolysis for 15 minutes. Based on these observation, an hydrolysis period of 20 minutes was adopted.

The material to analyzed was suspended in 6 ml of 5 per cent TCA and heated at 90°C for 20 minutes in water bath. After hydrolysis the material was centrifuged and the desoxypentose in the extract was estimated by the diphenylamine method.

2) Color development—Duplicate 2 ml. aliquots of the hydrolyzed material to be assayed were transferred to test tubes, and 4 ml of diphenylamine reagents were pipetted into each. The blank consisted of 2 ml of distilled water and 4 ml of the reagent. Tubes were heated for exactly 5 minutes in a boiling water bath, and then cooled immediately in cold water for 2 minutes. After 10 minutes, the blue solution was transferred to a 10 mm wide cuvette. The optical density was measured against a distilled water blank, by means of an A.K.A. photometer equipped with a No. 1 filter of wave-length 670 mμ. The concentration of DNA correspondng to this photometer reading was determined from a standard curve, obtained as descibed below.

Dische[37] originally used 3 minutes' heating. However, it is recognized by most authors that longer heating increases the intensity of the color, and it reaches a maximum after 10 minutes. After 10 minutes' treatment our samples showed a green color. The optical density of the samples increased but was very unstable. Therefore, a 5 minute heating period was employed in the present study.

3) Standarization of Test—In order to show that the amount of colored

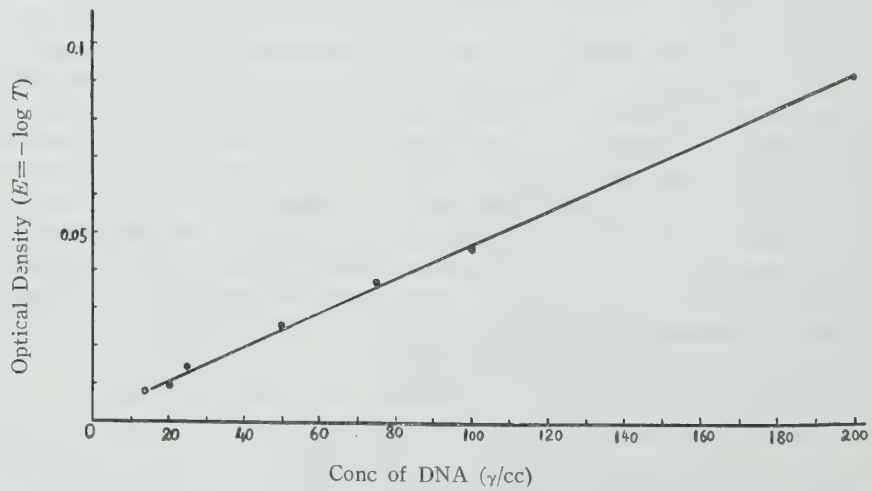


Fig. 3. Standarization Curve.

product formed with diphenylamine is directly proportional to the amount of DNA present, the color development procedure was applied to various dilutions of DNA standard soltution (10-200γ). The standard curve was obtained by plotting the amounts of DNA used against photometer values obtained by reading a series of tubes containing DNA against a blank containing only the reagent. The developed color followed Beer's law over the range of 15 to 200 γ of DNA (Fig. 3). In this study 40 to 120 mg of the dry powder, containing 40 to 130 γ of DNA, was examined for the diphenyl-

Table 10. DNA Content per Conidium and per Nuclus of Diploid Strain(G) and of Wild Type (K.S.) Strain of *Asp.sojae*.

Exp. No.	Wild type (K.S.)		Green Prototrophic Diploid (*y leu/al his*)	
	$10^{-7}\gamma$/Conidium	$10^{-7}\gamma$/Nucleus*	$10^{-7}\gamma$/Conidium	$10^{-7}\gamma$/Nucleus*
I**	—	—	2.72	1.27
	2.79	0.62	2.74	1.27
II	3.16	0.70	3.31	1.54
	3.16	0.70	3.17	1.45
III	2.42	0.54	2.63	1.22
	2.90	0.64	2.88	1.34
IV	3.65	0.81	4.01	1.87
	3.73	0.83	3.32	1.54
V	3.51	0.78	2.84	1.32
	3.62	0.80	3.54	1.64
VI	3.49	0.78	—	—
	3.63	0.81	3.73	1.73
VII	—	—	—	—
	3.46	0.77	2.68	1.20
Mean	3.29	0.73	3.13	1.46

* Number of Nuclei per Conidium: K.S. (4.50); G (2.15)

** Culture Media: M.M. (Czapek's sol.)—I~IV C.M. (Koji-extract)—V~VII

Analysis of Variance

Factors	V	D.F.	M.S.	F
between Samples	22.89	1	22.89	1.08
between Expts.	728.31	6	121.38	5.76*
Samples × Expts.	136.63	6	21.11	1.00
Totals	887.83	13		
between C.M. and M.M.	32.01	1	32.01	1.51
Intra Med.	696.30	5	159.26	7.54
Samples × Expts.	136.63	6	21.11	1.00
Totals	874.94	12		

lamine reaction.

4) Recovery of DNA added to dry powder—The recovery of DNA added to dry powder was tested. When 30, 60, and 120 γ of DNA were added to each sample the recovery of DNA ranged from 95 to 110 per cent of the amount added.

The relationship between the amounts of dry powder and the optical density readings was also examined. With increasing amounts, 1/2, 1/1, 2, and 3 times of dry powder, the optical density increased in direct proportion.

To determine the DNA content per conidium of the original wild type and of the stable green strain, 7 analyses were made in duplicate. 4 analyses were carried out for cultures grown on M. M. and 3 for cultures grown on C. M. The results are summarized on the basis of cell numbers

Table 11. DAN Content per mg Dry Weight* of Conidia of the Diploid Strain(G) and of the Wild type (K.S.) Strain of *Asp.sojae*.

Exp. No.	Wild type (K.S)			Green Prototrophic Diploid (*y leu/al his*)		
	DNA (γ)	Dry Weight (mg)	γ per mg Dry Weight	DNA (γ)	Dry Weight (mg)	γ per mg Dry Weight
I	39	56.3	0.693	43	38.7	1.111
	—	—	—	45	39.8	1.128
II	60	68.6	0.884	65	71.1	0.914
	60	70.6	0.849	60	67.7	0.886
III	41	45.0	1.111	50	44.8	1.138
	60	44.8	1.339	50	43.5	1.150
IV	129	114.9	1.122	69	47.2	1.462
	65	52.3	1.122	105	89.0	1.179
V	65	42.5	1.530	52	35.2	1.460
	65	44.5	1.460	65	35.6	1.600
VI	60	66.0	0.909	60	66.0	0.909
	60	57.3	1.045	—	—	—
VII	43	38.4	1.120	60	62.5	0.960
	—	—	—	—	—	—
Mean			1.098			1.158

* Dry weight obtained after extraction of acid soluble phosphates and of lipids.

Analysis of Variance

Factors	V	D.F.	M.S.	F
between Samples	2.93	1	2.93	0.36
between Expts.	224.17	6	37.36	4.62*
Samples × Expts.	48.53	6	8.09	1.00
Totals	275.63	13		

(Table 10). The range of variation between experiments and between samples as very small in duplicate runs.

There was no difference in DNA content per conidium between the green strains; however, the DNA content per nucleus of the supposed diploid green strain was twice as much as the wild type. Mean values per conidium were $3.29\times10^{-7}\gamma$ for the wild type and $3.13\times10^{-7}\gamma$ for the diploid green strain. These values do not agree with those given Heagy and Roper for the green strains of *Asp. nidulans*. The values reported by Heagy and Roper were stated to be in terms of 10^{-9} conidia. This would mean they found ca. $10^{-14}\gamma$ DNA per conidium. Perhaps there was an error in the units they reported. Yanagita[43] reports the occurrence of ca. $10^{-7}\gamma$ DNA per conidium of *Asp. niger* which value is similar to one found by us. As shown in the analysis of variance in Table 10, no significant difference of DNA content between cultures grown on C. M. and on M. M. was found.

Table 12. DNA Content per Conidium and per Nucleus of Yellow Segregant and of Yellow Mutant of *Asp.sojae*.

Exp. No.	L 120 (*y leu*)		Yellow Segregant* (*y*/*y*)	
	$10^{-7}\gamma$/Conidium	$10^{-7}\gamma$/Nucleus**	$10^{-7}\gamma$/Conidium	$10^{-7}\gamma$/Nucleus**
I	5.40	1.37	3.68	1.62
	4.57	1.16	4.31	1.89
II	3.35	0.85	4.68	2.06
	3.19	0.81	4.68	2.06
III	3.84	0.97	4.11	1.81
	4.07	1,03	4.37	1.92
IV	4.26	1.08	4.36	1.92
	4.26	1.08	4.38	1.97
Mean	4.12	1.05	4.32	1.90

* Yellow segregant from *y leu*/*al his* secured after UV in Exp. I in Table 4.

** Number of Nuclei per Conidium: *y leu* (3.94); *y*/*y* (2.27)

Analysis of Variance

Factors	V	D.F.	M.S.	F
between Samples	0.6481	1	0.6481	0.356
between Expts.	1.2520	3	0.4173	0.228
Samples × Expts.	5.4786	3	1.8262	1.00
Totals	7.3787	7		

Table 11 summarizes the data on the dry weight of conidia for both green strains. These were also nearly equal in DNA content per mg dry weight of conidia.

The values of DNA content per conidium of the yellow mutant and of

the yellow segregant from the heterozygous diploid green strain are based upon 4 analyses in duplicate of cultures grown on C. M. The mean values per conidium were $4.12\times10^{-7}\gamma$ for the yellow mutant and $4.32\times10^{-7}\gamma$ for the yellow segregant (Table 12). Just as in the two green strains, it was found that the yellow mutant and the yellow segregant were nearly the same in DNA content per conidium, but the DNA content per nucleus of the latter strain was almost twice that of the former strain. Whether the somewhat low DNA content of the "diploid" nuclei was due to an aneuploid condition can not be determined form the evidence available. Although the DNA content per nucleus confirms the evidence that the stable green strain and its yellow segregant are actually diploid, however, this conclusion may be not drawn on the basis of the DNA content per conidium. Heagy and Roper concluded that their green strain was diploid from the DNA content per conidium. As the diploid and haploid strains of *Asp. nidulans* have uninucleate conidia, we may also conclude that the DNA content per diploid nucleus was double that of the haploid. In *Asp. sojae*, however, the conidia of the wild type have an average of 4.50 nuclei and those of the supposed diploid strain, 2.15 nuclei, so the interpretation of data is more complicated.

DISCUSSION

The present study shows that new stable strains can be synthesized by producing heterozygous diploids from heterocaryons between two different strains. The spontaneous occurrence of stable green strains among the conidia of balanced heterocaryons was, however, exceedingly low ($0.2\text{–}3.0\times10^{-6}$ in *Asp. sojae*. and $0.25\text{–}3.6\times10^{-7}$ in *Asp. nidulans* and *P. chrysogenum* according to Pontecorvo *et al*). The difference between the two results almost disappears when the data for *Asp. sojae* are put on a per nucleus basis by dividing by 5, the number of nuclei per heterocaryotic conidium. *Asp. nidulans* and *P. chrysogenum* have uninucleate conidia and therefore, the data obtained with them require no correction. The reasons for this low frequency have not been resolved. It may be that nuclear fusions occur rarely or that the strength of selection for diploid nuclei during the growth of a colony is very low, etc.

The authors have succeeded, however, with ultraviolet light in producing stable green diploid strains in a considerable higher frequency ($2\text{–}9\times10^{-2}$). These strains were the same in genetic properties as the diploid strains appearing spontaneously. The enhancement of the frequency by ultraviolet treatment does not depend only upon selection of the stable green strains, because there was an absolute increase in their number. No experiments to elucidate whether nuclear fusion in the conidia of heterocaryons occurs directly, or indirectly through the transfer of nuclear material, could be carried out.

Gnetic analysis for color and for nutritional markers confirms that the new stable strains in a low frequency form recombinants and produce

segregants carrying one or more markers in homozygous form, although almost all retain the original combinations. In addition, a yellow segregant gave rise to further segregants like the stable green strains. It is therefore clear that the stable green strains and some segregants are heterozygous for some genetic markers. Most of the yellow segregants selected directly from the stable green strains were prototrophs, but some had nutritional requirements similar to the component strains. However, two segregants among them seemed homozygous for two factors. Furthermore, one segregant had the green conidial color of the heterozygous diploid but was auxotrophic. Unfortunately we have not as yet the proper data on segregants having these recombined properties of the two component strains to be sure of the mode of their origin. However, it may be suggested that the process of segregation takes place by somatic crossing-over as in the three filamentous fungi described by Pontecorvo *et al.* Special experiments to elucidate this matter will have to be conducted.

Another important problem is that of linkage. In this study loci for color markers and some nutritional markers have been identified. However, a survey for linkage between any two or more of these must be the subject of further research. For this purpose *Asp. sojae* is not optimal because of the impossibility of comparing the results of somatic recombination with those obtained by sexual reproduction, and because of the uncertainty of the cytology. Pontecorvo has shown, however, that somatic recombination alone may be used satisfactorily to map chromosomes and that it has the added advantage of allowing the centromere to be located.

Pontecorvo *et al* distinguished diploids from haploids in *Asp. niger* and *P. chrysogenum* by using the diameter of the conidia as a criterion. In the present study the stable green strains and their segregants were indistinguishable from the original wild type or the component strains in the diameter of their conidia. Differences between them were found, however, in the number of nuclei per conidium and in the DNA content per nucleus: The diploid strains had one half as many nuclei and twice as much DNA per nucleus as the haploid strains. These findings support the conclusion that the stable green strains and their yellow or albino prototrophic segregants are diploid. We have not yet observed the formation of haploid segregants from the diploid strains. The DNA content per conidium has considerable reliability as compared with the diameter of conidia alone because of its reproducibility and precision and because the diameter of the conidia does not reflect the state of ploidy when the conidia are multinucleate as in *Asp. sojae*. In the estimation of the DNA content, however, many sources of error are present: the extraction of DNA from conidia, the specificity of DNA for the colorimetric reaction, the precision of colorimetry, etc.

Studies on the relation of ploidy to radiation inactivation through survival-curve analysis have been made in yeast by many workers (Tobias[44]; Lucke and Sarachek[45-46]; Townsend, Sarachek and Lindegren[47]; Uretz[48];

Pomper, Daniels, and McKee[49]). They showed that the target value and rate of logarithmic inactivation are related to the DNA content in a complex way. Further experiments to dertermined diploidy or haploidy in the above strains will be done by using the survival-curve analysis, although *Asp. sojae* is much less suitable than uninucleate species of other filamentous fungi because of the multinucleate nature of its conidia.

Koji-molds are asexual fungi and therefore, the improvement of the strains has hithertofore depended upon selection, either of isolates from nature or of induced mutants. Heterocaryons were obtained fairly easily from nature or by mixing component strains. However, an efficient technique for the improvement of strains had not previously been worked out; the heterocaryons utilized by Lindegren and Andrews,[50] by Yuill [51], and Ciegler and Vezina[52] were unsatisfactory on account of their instability.

The technique described here has now been applied with consistent results to the production of stable strains of new genetic constitutions. Strain improvement may now be achieved by the formation of heterocaryons between two strains with known genetic markers and by UV treatment of the heterocaryons. We thus expect to find desirable properties in both the original stable diploid strains and their segregants. This improvement of strains will have very practical implications for industrial fermentation.

SUMMARY

1. The artificial production of new stable heterozygous diploid strains was carried out by using mutants of *Asp. sojae* with color and nutritional markers.

2. These new strains were obtained through the formation of heterocaryons in the following combinations: *y leu+al his*, *y arg+al his*, and *y arg +al leu*.

3. The frequency of the spontaneous occurrence of diploid strains differs according to the combinations of the component strains (0.2–3.0 G per 10^6 plated conidia).

4. The frequency of the occurrence of the diploids was raised to 2–9 per 10^2 surviving conidia by UV irradiation—this was an increase of $>10^4$ times.

5. The properties of the heterozygous diploids are as follows:

a) Conidial heads were uniformly green like the original wild type, while all heterocaryons had, in varying proportions, mixtures of white, yellow, and green conidial chains.

b) Growth on M. M. was like that of heterocaryons but unlike either component strain. On C. M., or a synthetic medium supplemented with either or both of the growth factors required by the component strains, they exhibited mostly homogenous green colonies, instead of segregating out into yellow and albino sectors like the heterocaryon.

c) They were exceedingly stable and 99.5 per cent of the colonies remained green. After 10–14 days' incubation, they rarely produced spots or sectors of either component color. The majority of the heterocaryons segregated into the two component strains, however, and only about 15 per cent remained heterocaryotic.

d) Genetic analysis of the diploids showed the following results. As for the color markers, 0.1–0.5 per cent of yellow or albino segregants occurred. Among these, yellow segregants further gave albinos in low ratio (0.1–0.3 per cent). Concerning the nutritional marker, the diploids were prototrophic and yellow or albino segregants were either prototrophic or required the same substances as the component strains. However, in the combination *y arg+al leu*, three new segregants have been obtained which differ from the component strains.

e) The diploids and their segregants were nearly the same as the original wild type and the component strains in the diameter of their conidia.

f) Heterozygous diploids and their segregants had on the average about one half as many nuclei as the original wild type or the component strains.

g) The DNA content per conidium was nearly the same as the original wild type and the component strain, but the DNA content per nucleus was about twice as large.

h) The new efficient method developed for the production of new strains of asexual fungi promises much in the way of the strain improvement required by industry.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the valuable advice and criticism of Professor B. Wada and Professor F. J. Ryan, and also the contribution to the estimation of DNA made by Mr. K. Uchida.

REFERENCES

(1) Ishitani, C. and Sakaguchi, K.: *J. Gen. Appl. Microbiol.* **2**, 345 (1956).
(2) Roper, J. A.: *Experimentia* **8**, 14 (1952).
(3) Pontecorvo, G.: *Nature* **170**, 204 (1952).
(4) Pontecorvo, G. and Roper, J. A.: *Advance in Genet.* **5**, 218 (1953).
(5) Pontecorvo, G., Roper, J. A., and Forbes, E.: *J. Gen. Microbiol.* **8**, 198 (1953).
(6) Pontecorvo, G. and Sermonti, G.: *Nature* **172**, 126 (1953). *J. Gen. Microbiol.* **11**, 94 (1954).
(7) Sermonti, G.: *Rend. Ist. Sup. Sanita* **17**, 231 (1954).
(8) Ishitani, C.: *J. Agr. Chem. Soc. Japan* **29**, 682 (1955).
(9) Ishitani, C., Ikeda, Y. and Sakaguchi, K.: *J. Gen. Appl. Microbiol.* **1**, 272 (1955).

(10) IGUCHI, N.: *J. Agr. Chem. Soc. Japan* **23**, 16 (1949).
(11) : *ibid.* **25**, 459 (1952).
(12) LEVAN, A.: *Nature* **158**, 626 (1946).
(13) : *Hereditas* **13**, 31 (1947).
(14) : *ibid.* **24**, 471 (1938).
(15) SANSOME, E. R.: *Trans. Brit. Mycol. Soc.* **32**, 306 (1949).
(16) ATWOOD, K. C.: *Genetics* **37**, 564 (1952).
(17) ——: *Proceeding Int. Cong. Phytobiol. Amsterdum* 1954.
(18) HASS, F., WYSS, O., and STONE, W. S.: *Proc. Nat. Acad. Sci.* **34**, 229 (1948).
(19) HAYES, W.: *Nature* **169**, 1017 (1952).
(20) JAMES, A. P.: *Genetics* **40**, 204 (1955).
(21) PONTECORVO, G.: *Caryologia Vol. Suppl.* 1 (1954).
(22) PONTECORVO, G., GLOOR, E. T., and FORBES, E.: *J. Genetics* **52**, 226 (1954).
(23) FRIES, N.: *Hereditas* **34**, 338 (1948).
(24) : *Physiol. Planter.* **1**, 330 (1948).
(25) LEIN, J., MITCHELL, H. K. and HOULAHAN, M. B.: *Proc. Nat. Acad. Sci.* **34**. 435 (1948).
(26) WOODWARD, V. W., DE ZEEUW, R., and SRB, A. M.: *ibid.* **40**, 192 (1954).
(27) CATCHESIDE, D. G.: *J. Gen. Microbiol.* **11**, 34 (1954).
(28) FORBES, E.: *Microbial Genet. Bull.* **6**, 26 (1952).
(29) RAPER, J. A. and PRITCHERD, R. H.: *Nature* **175**, 639 (1955).
(30) MUNDKUR, B.: *Experimentia* **9**, 373 (1953).
(31) HUEBSCHMAN, C.: *Mycologia* **44**, 599 (1952).
(32) VENDRELY, R., and VENDRELY, C.: *Experimentia* **4**, 434 (1948).
(33) BOIVIN, A., VENDRELYR, R., and VENDRELY, C.: *Compt. Rend.* **226**, 1061 (1948).
(34) MIRSKY, A. E. and RIS, H.: *Nature* **163**, 666 (1949).
(35) HEAGY, F. C. and ROPFR, J.A.: *idid.* **170**, 713 (1952).
(36) OGUR, M., MINKLER, S., LINDEGREN, G., and LINDEGREN, C. C.: *Arch. Biochem.* and *Biophys.* **40**, 175 (1952).
(37) DISCHE, Z.: *Microchemie* **8**, 4 (1930).
(38) SEIBERT, F. B.: *J. Biol. Chem.* **133**, 593 (1940).
(39) SCHNEIDER, W. C.: *ibid.* **161**, 293 (1945).
(40) STEELE, R., THEODORE, S., and OTTOLENGHI, L.: *ibid.* **177**, 231 (1949).
(41) CHARGAFF, E. and DAVIDSON, J. N.: *The Nucleic Acids* **Vol. 1**. (1955).
(42) WEBB, J. M. and LEVY, H. B.: *J. Biol. Chem.* **312**, 107 (1955).
(43) YANAGITA, T.: *Arch. Mikrobiol.* in Press.
(44) TOBIAS, C. A.: *Symposium on Radiology* 357 (1950).
(45) LUCKE, W. H. and SARACHEK, A.: *Nature* **171**, 1014 (1953).
(46) and : *Arch. Biochem and Biophys.* **44**, 271 (1953).
(47) TOWNSEND, G. F., SARACHEK, A., and LINDEGREN, C. C.: *Cytologia* **19**, 130 (1954).
(48) URETZ, R. B.: *Radiation Research* (1955).
(49) POMPER, S., DANIELS, K. M., and MCKEE. D. W.: *Genetics* **39**, 343 (1954).
(50) LINDEGREN, C. C. and ANDREWS, H. N.: *Bull. Torroy Bot. Club.* **72**, 36 (1945).
(51) YUILL, J. L.: *J. Gen. Microbiol.* **9**, 1 (1953).
(52) CIEGER, A. and VEZINA, C. E.: *Bact. Proc.* 30 (1955).

J. Gen. Appl. Microbiol.
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HIOCHIC ACID, A NEW GROWTH FACTOR FOR *LACTOBACILLUS HOMOHIOCHI* AND *LACTOBACILLUS HETEROHIOCHI*

(Preliminary Report)

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Received for publication Aug. 1, 1956

A new growth factor indispensable for the growth of "True Hiochi bacteria"[1] which were recently named as *Lact. homochiochi* and *Lact. heterohiochi*[2] was isolated and purified. The Sake (Japanese rice wine) which is frequently spoiled by the "Hiochi" bacteria, contains this factor. It was not detected in liver extract, yeast extract, tomato juice, whey and laboratory peptones, but was found to be considerably accumulated in the culture-broth of several organisms, such as *Aspergillus*, *Penicillium*, *Monilia*, *Willia* and *Lactobacillus*. By means of bioautography it was confirmed that the "Hiochi" factors produced by these organisms in the broth were all the same[3].

From the broth in which *Aspergillus oryzae* was grown, the "Hiochi" factor was extracted with butanol and/or ethylacetate after being acidified to pH 2.0. A highly purified barium salt of the factor was obtained by the chromatographic technique using silica gel[4,5]. When the barium salt was acetylated with acetyl chloride or acetic anhydride, its activity for growth disappeared completely. However, the activity was recovered after alkali-hydrolysis of the acetyl derivative, a fact indicating the presence of hydroxyl group.

The p-bromophenacylester was not obtained in crystalline form, whereas crystallization was succeeded for the quinine salt and the S-benzylthiuromium salt (Fig. 1).

Analyses of these two salts gave the following results.

Quinine salt; m.p. 137–8°C.

Anal. Calculated for $C_{26}H_{36}O_6N_2$: C, 66.08; H, 7.65; N, 5,93. Found: C, 66.01,66.17; H, 7.64, 7.51; N, 5.92.

S-benzylthiuronium salt; m.p. 122–3°C.

Anal. Calculated for $C_{14}H_{22}O_4N_2S$: C, 53.49; H, 7.05; N, 8.91; S, 10.18, Found: C, 53,13, 53.41; H, 7.11, 7.16; N, 9.05; S, 9.49.

The "Hiochi" factor freed from its quinine salt was then distilled in vacuo. A viscous syrupy material obtained showed the following charac-

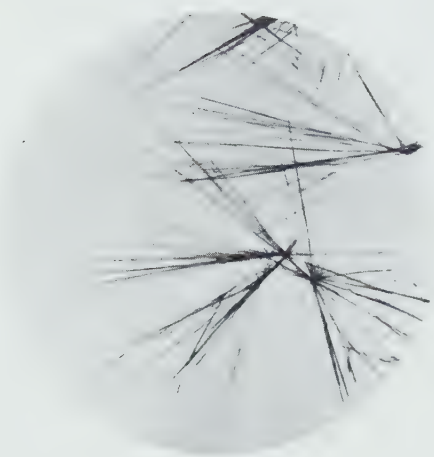


Fig. 1. Crystal of Quinine salt of Hiochic acid. magnification × 200

teristics: b.p. 5 mm 145–50°C, b.p. 2 mm 130–35°C, $n^{19}=1.692$.

$[\alpha]_D^{20}-19.9°$ (C, 3.47 in 95% alcohol)

Anal. Calculated for $C_6H_{10}O_5$: C, 55.37; H, 7.75; Found: C, 55.20, 55.23; H, 8.00,7.81.

The titration curve of the distilled material showed a sharp rise in pH followed by a gradual drop. The hydroxamic acid test proved positive for the material. These two facts suggest the presence of a lactone ring.

By the back titration the pKa value was found to be 4.38 which is intermediate between the values of an unsubstituted carboxylic acid and an alpha-hydroxy acid. On the other hand, the alkaline periodate test was negative showing the absence of adjacent hydroxyl groups.

When the lactone was heated in 20% H_2SO_4, the ultraviolet absorption at 222 mμ increased considerably to attain an ε value of about 7,000. The heated substance consumed permangnate at room temperature. It is clear, therefore, that the lactone was converted to α-β-unsaturated acid by the sulfuric acid treatment. This fact indicates that the "Hiochi" factor is a beta-hydroxy acid.

No colour was developed upon the addition of ferric chloride to the aqueous solution. The aldehyde and ketone group test using sodium nitroprusside, silver mirror reaction, or the Fehling solution, were all negative. The tetranitromethene test and the bromine absorption test for olefinic unsaturation were equally negative. The absence of unsaturated carbon bond and aldehyde-ketone group was also clearly evidenced by the structure of infrared spectra (Fig. 2 and 3).

The infrared spectrum of barium salt of "Hiochi" factor in Nujor mull, indicated the presence of an ionized carboxyl group (6.37 μ) and a hydroxyl group (3.0 μ). The infrared spectra of the distilled syrupy lactone, which were measured using a liquid film (Fig. 2) and a chloroform solution of the material (Fig. 3) showed clearly the presence of δ-lactone (5.80 μ) as well as the presence of C-CH_3 (6.78 μ and 7.24 μ), and a hydroxyl group (liquid film, 2.92 μ; chloroform solution, 2.90 μ and 2.76 μ). Moreover, it was deduced, from the OH-deformation and the C-O stretching absorption in the spectra (Figs. 2 and 3), that the hydroxy group would be that of secondary or tertiary alcohol.

It is concluded, therefore, that the "Hiochi" factor is a saturated β-hydroxy

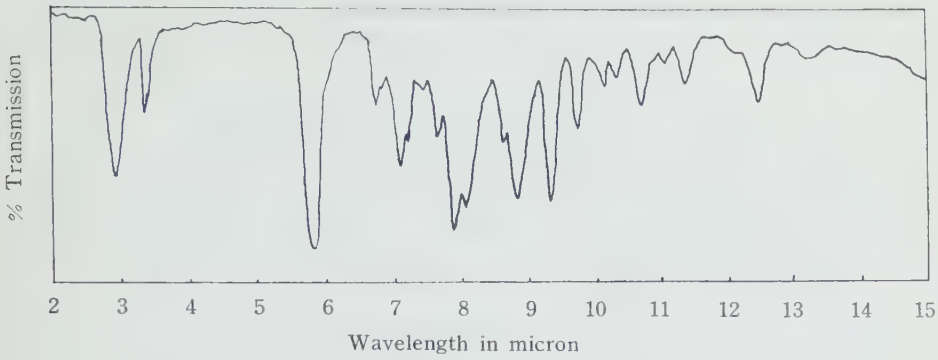


Fig. 2. Infra red spectrum of the lactone of Hiochic acid in liquid film.

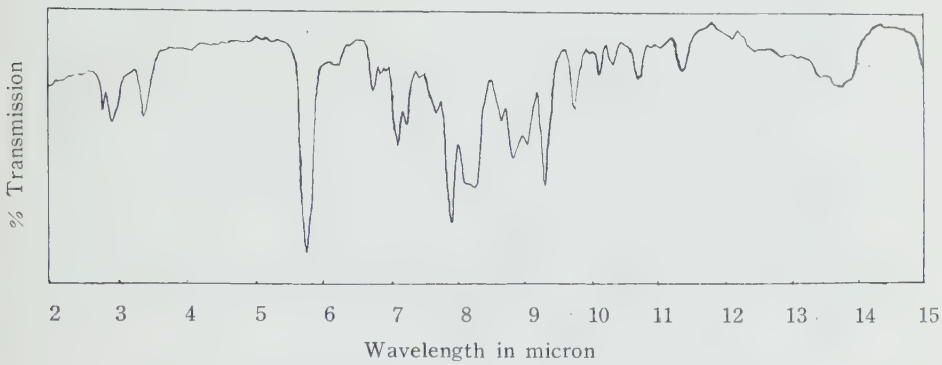
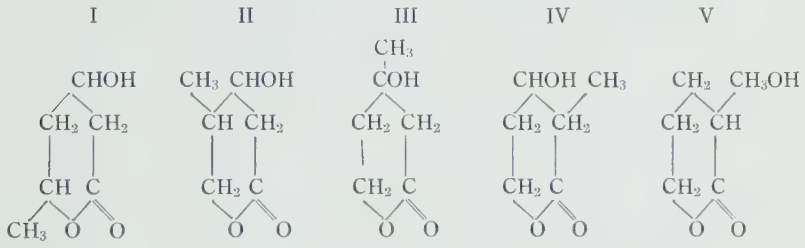


Fig. 3. Infra red spectrum of the lactone of Hiochic acid in chloroform.

δ-lactone having a formula of $C_6H_{10}O_2$. The following four structures (I to IV) are compatible with the observations described above[6].



Among these possibilities, structure I seems to be the most probable in view of the fact that the iodoform formation test for $-CHOH-CH_3$ group with alkaline iodine was distinctly positive.

The "Hiochi" factor, in its lactone form and as barium salt supports the maximum growth of *Lact. heterohiochi* at the concentration of 0.2 μg

and 0.35 μg per ml respectively of the medium. It is proposed to designate the "Hiochi" factor by the name "Hiochic Acid."

ACKNOWLEDGEMENT

The author wishes to express his sincere thanks to Professor Kin-ichiro Sakaguchi for his guidance throughout this work. Thanks are also due to Professor Kyosuke Tsuda and to the members of the laboratory of Professor Yusuke Sumiki for their kind advice and suggestions. A large scale cultivation was carried out through the courtesy of Meiji Seika Co. Ltd.

REFERENCES

1) Teizo Takahashi; Report of the Governmental Institute of Brewing, Vol. 12, (1906), Vol. 59, (1915)
2) Kakuo Kitahara et al., Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, March 30, 1955.
3) Gakuzo Tamura; Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April 5, 1952.
4) Gakuzo Tamura; Presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April 7, 1953.
5) Kin-ichiro Sakaguchi; Annual Report of Brewers Research Vol. 11, 115, 1955 published by Brewer's Association (Jôyukai)
6) Kin-ichiro Sakaguchi; Presented at the Annual Meeting of the Brewers Association. June 1956.
Annual Report of Brewer's Research Vol. 11, 108, 1956.
Published by Brewer's Association (Jôyukai)

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THE JOURNAL OF GENERAL AND APPLIED MICROBIOLOGY

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Printed by
Kokusai Bunken Insatsusha
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